THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

C. R. HARINGTON

ASSOCIATE EDITORS

S. J. COWELL

F. DICKENS

and

F. J. W. ROUGHTON

VOLUME XXXV 1941

PART II, pp. 749 to end

CAMBRIDGE

AT THE UNIVERSITY PRESS

1941

Vol. 35, No. 7

DEC 1 1941

July, 1941

Medical Library

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BENTLEY HOUSE, N.W. 1

CHICAGO: The University of Chicago Press

(Agents for the United States)

BOMBAY, CALCUTTA, MADRAS: Macmillan

TOKYO: Maruzen Company, Ltd.

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1941

Price Seven Shillings and Sixpence net

PRINTED IN GREAT BRITAIN

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79. THE BEHAVIOUR OF *SCOPULARIOPSIS BREVICAULIS* TOWARDS CHOLINE

By CHARLES SIMONS

From the Organic Chemistry Department, The University, Leeds

(*Received* 23 *April 1941*)

CHOLINE (I) and its lower homologue colamine (IV) are known to occur in nature, combined, as in the phosphatides or lecithin [Schulze & Likiernik, 1891; Trier, 1911; Baumann, 1913; McLean, 1908, 1, 2]. Choline is also known to occur simply as cyclic choline sulphate, in the micro-organism *Aspergillus sydowi* [Woolley & Peterson, 1937] and also in the hepatic bile [Johnston *et al.* 1939]. In most respects choline is considered to be a vital cell ingredient. No report has been made of the natural occurrence of the two intermediate members *NN*-dimethylaminoethanol (II) and *N*-methylaminoethanol (III).

$HO.(CH_3)_3N.CH_2.CH_2.OH$ (I) $(CH_3)_2N.CH_2.CH_2.OH$ (II)

$CH_3.NH.CH_2.CH_2.OH$ (III) $NH_2.CH_2.CH_2.OH$ (IV)

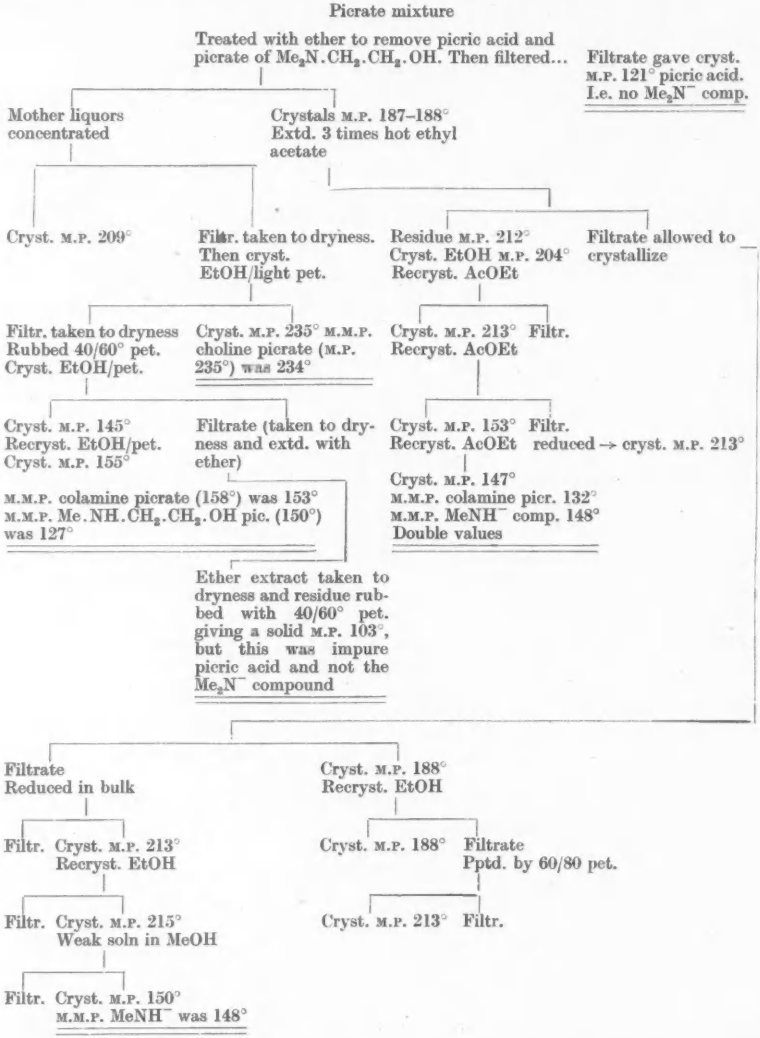
The methylation of As_2O_3 to trimethylarsine by *Scopulariopsis brevicaulis* has been discussed by Challenger *et al.* [1933; 1935; 1936]. Challenger & Higginbottom [1935] suggested that choline or betaine might be the source of the methyl group. A similar hypothesis had already been put forward by Riesser [1913] when considering the origin of the methyl group of creatine, and as an explanation of the garlic odour (assumed to be dimethyl telluride) exhaled by animals on ingestion of tellurite. Riesser's suggestion has, however, been considerably extended by the Leeds workers. It is conceivable that choline acts as a methylating agent while being converted into any or all of its three lower homologues mentioned above. Now, the amount of choline and related amino-alcohols present in a micro-organism such as a mould of the *Penicillium* or *Aspergillus* type would be small and difficult to detect. It would be advantageous therefore, to incorporate choline chloride in the arsenical medium in the hope that when detoxication (i.e. biological methylation) took place, the demethylation products would remain in the medium if the reaction were an extracellular enzymic one or diffuse into the medium if the reaction were intracellular. In this way a reasonable amount of amino-alcohols might be available for detection.

Scopulariopsis brevicaulis (sometimes called *Penicillium brevicaule*) was grown for several weeks upon a medium incorporating inorganic salts in glucose solution containing choline chloride (series A). To some was added As_2O_3 (series B) and controls were set up on media containing neither choline nor arsenic (series D) and containing arsenic but no choline (series C). These last two were used to see the effect of medium on picrate formation.

Before investigation of the media could be carried out an ideal separation of the four aminoethanols (I, II, III, IV) had to be devised, as it is possible that demethylation could stop at any of the stages and thus produce all four.

Various compounds of the bases are described in the literature, e.g. the mercurichloride, chlorplatinate, reineckate, picrate, picrolonate, chloraurate, iodobismuthate, phosphomolybdate, phosphotungstate etc. [Guggenheim, 1920;

Kahane & Levy, 1938; Ladenburg, 1881; Knorr, 1889; Knorr & Matthes, 1898; 1901; Matthes, 1901] and it was hoped that by a judicious selection of reagents the presence of each base in a mixture could be ascertained. The similarity in properties, however, of these salts and compounds precluded this course and it was finally decided to use fractional crystallization methods on one series of salts. Trials on the picrates, picrolonates and mercurichlorides showed that the former were the best. The separation was then applied to the biological media upon which the mould had been growing. Only choline was detected.


Picrate mixture
Treated with ether to remove picric acid and picrate of $Me_2N.CH_2.CH_2.OH$. Then filtered...
Filtrate gave cryst. M.P. 121° picric acid. I.e. no Me_2N^- comp.
Mother liquors concentrated
Crystals M.P. 187–188° Extd. 3 times hot ethyl acetate
Cryst. M.P. 209°
Filtr. taken to dryness. Then cryst. EtOH/light pet.
Residue M.P. 212° Cryst. EtOH M.P. 204° Recryst. AcOEt
Filtrate allowed to crystallize
Filtr. taken to dryness Rubbed 40/60° pet. Cryst. EtOH/pet.
Cryst. M.P. 235° M.M.P. choline picrate (M.P. 235°) was 234°
Cryst. M.P. 213° Filtr. Recryst. AcOEt
Cryst. M.P. 145° Recryst. EtOH/pet. Cryst. M.P. 155°
Filtrate (taken to dryness and extd. with ether)
Cryst. M.P. 153° Filtr. Recryst. AcOEt
reduced → cryst. M.P. 213°
M.M.P. colamine picrate (158°) was 153° M.M.P. Me.NH.CH_2.CH_2.OH pic. (150°) was 127°
Cryst. M.P. 147° M.M.P. colamine picr. 132° M.M.P. $MeNH^-$ comp. 148° Double values
Ether extract taken to dryness and residue rubbed with 40/60° pet. giving a solid M.P. 103°, but this was impure picric acid and not the Me_2N^- compound
Filtrate Reduced in bulk
Cryst. M.P. 188° Recryst. EtOH
Filtr. Cryst. M.P. 213° Recryst. EtOH
Cryst. M.P. 188° Filtrate Pptd. by 60/80 pet.
Filtr. Cryst. M.P. 215° Weak soln in MeOH
Cryst. M.P. 213° Filtr.
Filtr. Cryst. M.P. 150° M.M.P. $MeNH^-$ was 148°

Experimental

NN-Dimethylaminoethanol was prepared by the method of Ingold & Rogers [1935], *N*-methylaminoethanol by that of Slotta & Behnisch [1932] and colamine by that of Ing & Manske [1926]. Choline chloride was purchased from the British Drug Houses Ltd.

Approximately equal amounts of choline chloride and the other three bases were mixed and made acid to Congo red with HCl and reduced to a syrup on the steam bath. $NaHCO_3$ solution was then added in excess to make 5% excess $NaHCO_3$ and KI solution (153 g. I_2 and 100 g. KI in 200 ml. water) added until precipitation was complete and the mixture kept overnight. This removed most of the choline as the insoluble ennea-iodide [Stanek, 1905]. The dark precipitate was filtered and sparingly washed. To the filtrate was added dilute HCl and then $CuCl_2$ and 'molecular' Cu and the whole boiled. This was continued until the solution had a permanent blue colour. It was then filtered and again heated and all the excess Cu precipitated as the sulphide by H_2S and again filtered. The colourless filtrate was reduced in bulk on the steam bath and $NaHCO_3$ added until the solution was only just acid to Congo red. The whole was then taken to dryness on the steam bath and when cold was extracted with cold methanol several times and filtered. The solution was then made to react with saturated alcoholic sodium picrate and filtered.

The significance of the recurrence of the melting point 213° would indicate a eutectic of choline and methylaminoethanol.

As it was not found possible to get a good separation of the dimethylaminoethanol from the ether extract of the picrates it was identified as follows.

Benzyl bromide and dimethylaminoethanol react in dry ether solution to give a white hygroscopic solid of the quaternary salt, M.P. 128° (cryst. alc. ether). Br, found 30·38%. Calc. for $C_{11}H_{18}ONBr$ 30·56%. Colamine and the monomethyl compound do not react so; hence it is possible to identify the dimethyl compound in a mixture of all three by dissolving the free bases in dry ether, adding benzyl bromide and seeding with a few crystals of the pure quaternary salt whereupon the oil crystallizes, M.P. 121°, M.M.P. 122°.

Application to biological media

Thirty-six 1 l. conical flasks, each containing the following substances, were set up and sterilized.

	Series A	Series B	Series C	Series D
No. of flasks	12	12	6	6
Czapek-Dox salt solution*	200 ml.	200 ml.	200 ml.	200 ml.
Choline chloride	2 g.	2 g.	Nil	Nil
Glucose	4 g.	4 g.	4 g.	4 g.

* Czapek-Dox salt solution [cf. Chalmers, 1935]:

$NaNO_3$	2 g.
KH_2PO_4	1 g.
KCl	0·5 g.
$MgSO_4$	0·5 g.
$FeSO_4$	0·01 g.
Water to	1 l.

All 36 flasks were inoculated from Czapek-Dox-sucrose agar slopes of *Scopulariopsis brevicaulis* (strain B) [Challenger *et al.* 1933], incubated for 3 days at 32° and then allowed to stand at room temperature.

After 21 days, to each of series B and C were added 25 ml. of a sterile 1·2% As_2O_3 solution and the flasks again kept for some time. Series A and D were

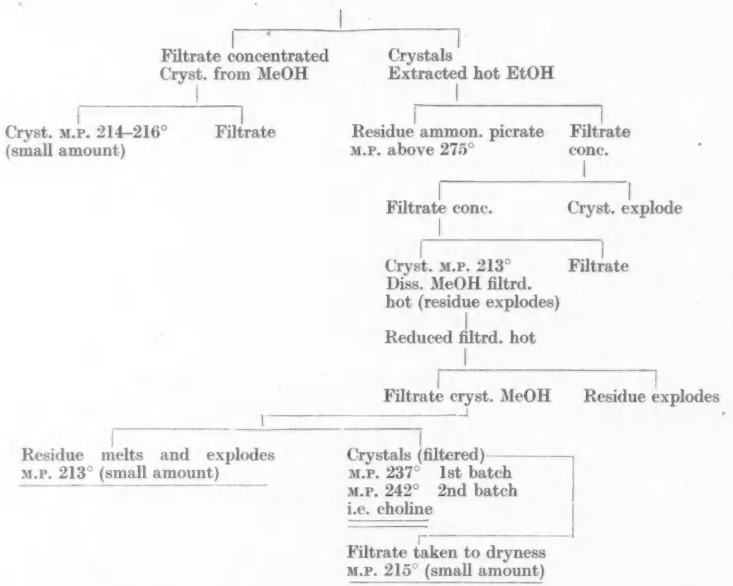
filtered after 91 days and series B and C after 147 days. All filtrates were concentrated, it being noticed that those from series A and B smelt strongly of what was considered to be trimethylamine.

1. The filtrate from series A was made alkaline with NaOH and extracted with $CHCl_3$, but very little was taken out by the solvent which was removed and the residue treated with picric acid. No picrate was obtained.

2. 10–20 ml. of series C filtrate were mixed with 0·5 g. choline chloride and made to react with 20–30 ml. of saturated alcoholic sodium picrate and then taken to dryness on the steam bath. Cryst. from MeOH M.P. 228°.

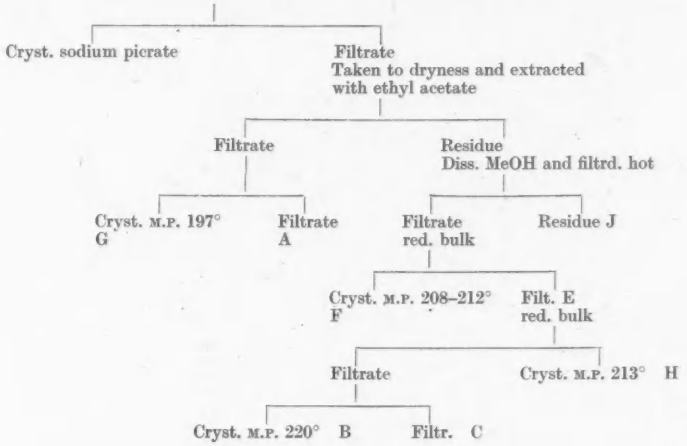
3. Repeated above for series D filtrate with similar results giving cryst. M.P. 231°.

4. Series A filtrate was acidified to congo red with HCl and taken to dryness. The residue was dissolved in alcohol and made to react with alcoholic sodium picrate and then reduced in bulk and filtered from colourless inorganic salts. It was washed with methanol, concentrated and filtered:

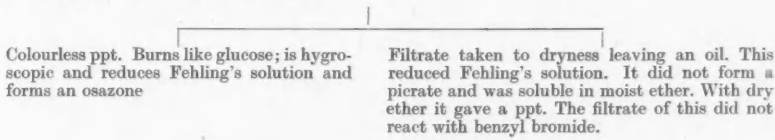


5. Some of the filtrate from series B was made acid to Congo red and taken to dryness. Aqueous sodium picrate (saturated) was added without filtering and the whole taken to dryness. It was heated with norite in MeOH and filtered hot giving a filtrate which deposited yellow crystals on cooling.

Residues A, B, C, E, F, G, H, J were collected, dissolved in water, acidified with HCl and extracted with ether to remove picric acid. The aqueous layer was boiled free from ether and made alkaline with $NaHCO_3$. The choline was then removed as the ennea-iodide and the filtrate treated with the usual reagents to remove the excess I_2 and KI, Cu etc. and finally the solution was made just acid to Congo red.



It was then reduced to dryness on the steam bath. The residue was extracted with cold MeOH three times and the liquid decomposed with saturated alcoholic sodium picrate. The residue was mainly sodium picrate. The liquid was then reduced in bulk and ether added.


Colourless ppt. Burns like glucose; is hygroscopic and reduces Fehling's solution and forms an osazone
Filtrate taken to dryness leaving an oil. This reduced Fehling's solution. It did not form a picrate and was soluble in moist ether. With dry ether it gave a ppt. The filtrate of this did not react with benzyl bromide.

The conclusions that may be drawn therefore from the foregoing are that choline is not broken down completely by the organism and that, if any is broken down, it is converted either into ammonium salts, trimethylamine or some other nitrogenous product, or that the compounds for which search was made are bound up in the mycelial portion as an essential cell constituent, possibly combined, for example, as a phosphatide.

Summary

1. A possible mechanism of the biological methylation of As_2O_3 by fungi such as *Scopulariopsis brevicaulis* has been suggested with choline as the methylating substance.

2. A means of identifying the possible products formed has been worked out, utilizing a picrate separation and the formation of a quaternary ammonium salt.

3. Application of this separation has shown that demethylation of choline to its lower homologues does not occur in the medium.

The author wishes to thank the Institute of Chemistry for the award of the Pedler Scholarship by which means this research was carried out and also Prof. F. Challenger for his advice and guidance.

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80. ASCORBIC ACID OXIDASE FROM CUCUMBER

By GEORGE TURNER MEIKLEJOHN and CORBET PAGE STEWART

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(*Received 6 June 1941*)

The oxidation of ascorbic acid by plant tissues (*in vitro*) was observed by Sz. Györgi [1928] who later postulated an enzyme responsible for this oxidation which might be connected with the function of the vitamin *in vivo*. The same general phenomenon was observed by many other investigators who used widely different plant tissues, and the dynamics of the reaction and its relationship to glutathione were worked out by Hopkins & Morgan [1936] who used the pressed juice from cauliflower as a source of the enzyme.

A certain amount of confusion has existed, however, since Barron *et al.* [1935] showed that ascorbic acid was not autoxidizable at *p*H below 7·6 and that the oxidation was catalysed by minute traces of Cu present in the solutions as impurity. Examination of the Cu content of vegetable juices showed that the oxidation of ascorbic acid by these juices could be accounted for mainly by the Cu. Haemochromogens also act as catalyst for this oxidation. McCarthy *et al.* [1939] further showed that the so-called natural enzyme activity could be imitated by loose combinations of Cu ions and protein and suggested that the vegetable juices, which contain both Cu and protein, oxidized ascorbic acid by virtue of the mutual presence of these two factors and, consequently, there was no need to postulate the existence of a true ascorbic acid oxidase. It should be noted, however, that the amounts of Cu present in the synthetic 'enzymes' reported by these authors were in excess of the amounts of Cu in the ionic form required to catalyse the reaction at the same rate under similar conditions: in other words, protein, while imposing upon the Cu ions the qualities of an enzyme, hindered rather than accelerated the catalysis. As will be shown later, ascorbic acid oxidase is much more active catalytically than the same amount of Cu in the ionic form. The Cu in the synthetic enzymes, moreover, was all removed by dialysis against water, a treatment which only removed part of the Cu from vegetable juices. A certain amount of the catalytic oxidation of ascorbic acid by plant juices is probably due to the presence of Cu ions but, as Spruyt & Vogelsgan [1938] have pointed out, this is insufficient completely to account for the oxidase activity.

Kubowitz [1938] isolated from potato an enzyme which oxidized polyphenols and which, in the pure state, contained some 0·2% Cu which was removed by dialysis against dilute acid but not water. Kubowitz also succeeded in reconstituting the enzyme 'molecule' by adding to a Cu-free, inactive enzyme preparation an inorganic Cu salt. More recently, Lovett-Janison & Nelson [1940] isolated from crook-neck squash an enzyme which contained 0·15% Cu, was free from peroxidase activity, and which catalysed the oxidation of ascorbic acid to a marked degree without showing significant activity towards phenols. The method used by these authors to express the activities of their preparations makes it difficult to find a suitable basis for comparison with the oxidase from

cucumber described here, so that no opinion can be advanced about the possible identity of the two enzymes. Both, however, show great catalytic activity in the oxidation of ascorbic acid, both contain Cu in amounts related to these activities, and both fail to catalyse the oxidation of polyphenols to a significant extent.

The experiments described here provide evidence for the existence in cucumber tissue of a discrete ascorbic acid oxidase in which the prosthetic group is Cu.

Experimental

The reaction rate for the oxidation of ascorbic acid was measured in the Warburg manometric apparatus using reaction vessels of the single side-bulb type. The reaction flasks each contained 4·20 ml. liquid: 0·20 ml. 10% KOH in the centre cup with filter paper; 0·20 ml. ascorbic acid solution containing 25 μ-mol. in the side bulb; and 3·80 ml. in the main space. 2·00 ml. buffer solution, 1·00 ml. portions of enzyme preparation, 0·20 ml. portions of suitable dilutions of other reagents such as diethyldithiocarbamate or $CuSO_4$ were used and the volume in the main space was made up to 3·80 ml. with distilled water. The buffer solutions used were acetate (M), McIlvaine's phosphate-citrate ($M/10$) and Clarke's phosphate ($M/4$), all at pH 6·0, the accuracy being tested in the Beckmann pH meter.

The reaction was started by tipping the ascorbic acid solution into the main space after temperature equilibration had been reached at 25°. Reaction rates were calculated from the initial, linear O_2 uptakes. The levels of the manometers were read every 5 min. after tipping except in the case of extremely small O_2 uptakes when the time interval was suitably increased. Control flasks containing (1) ascorbic acid and buffer solution, and (2) all reagents except ascorbic acid, were set up each time and the pH of the contents of each flask was checked after the reaction had been completed.

Cu-free distilled water was prepared by distilling water obtained from a laboratory electric still successively from $K_4Fe(CN)_3 + Ba(OH)_2$, and $KMnO_4 + Ba(OH)_2$. The distillate was then distilled several times from an all-glass pyrex still incorporating a 12-bulb fractionating column until a test run with reagents prepared from this water showed an O_2 uptake of less than 5 μl. per hr. with 25 μ-mol. of ascorbic acid at pH 6·0, in acetate buffer. Acetate buffer was used for the test since with it no inhibition of Cu catalysis occurs, as it does slightly with phosphate buffer. Similar precautions were taken with all reagents employed: ascorbic acid solutions prepared under these conditions were quite stable and the titre remained constant over long periods of time.

Cucumber juice was prepared by autolysing cucumber tissue. A cucumber was frozen overnight in a freezing mixture in the ice-chest before being sliced. The portions were placed in the neck of a large filter funnel above a porcelain grid or a loose plug of cotton wool and were allowed to thaw; the juice was collected in a pyrex bottle and stored in the ice chest. This method of preparation yielded a larger volume of clearer juice than any other tried, and avoided the necessity of further treatment to remove extraneous matter.

Dialysis of the juice was carried out in cellophane sacs of unknown porosity against Cu-free water at 0° and every care was taken to prevent contamination of the reagents. The dialysate was changed every 24 hr. and the ratio of the volume of cucumber juice to the volume of dialysate was kept constant at 1 : 4 since the detection and determination of Cu dialysed from the juice by means of the rate of catalytic oxidation of ascorbic acid depended upon this ratio. 4 volumes of dialysate were equivalent to 1 volume of juice: the total volume of

reactants in each Warburg flask was 4·00 ml. and the standard volume of enzyme preparation used was 1·00 ml. Thus, if the reagents for one flask were made up from a particular dialysate, the rate of oxidation of the ascorbic acid would depend on the amount of Cu in 4·00 ml. of dialysate, i.e. on the amount of Cu from 1·00 ml. juice being dialysed. The rates of O_2 uptake for 1·00 ml. enzyme preparation and 4·00 ml. dialysate were thus directly comparable without further manipulation. Volume changes in the dialysing sacs were small enough to be neglected.

The Cu in the enzyme preparations was determined by first evaporating the juice in a platinum basin at 60–70° and incinerating the residue in a platinum crucible, great care being taken to prevent loss of material by spurting. The ash was then taken up in 2×2 ml. Cu-free glacial acetic acid with evaporation to dryness after each addition; the residue was taken up in water and made up to the original volume of the enzyme preparation. The amount of Cu was determined in these solutions both by the rate of oxidation of ascorbic acid and colorimetrically in matched 50 ml. Nessler tubes with the sodium diethyldithiocarbamate reagent. Nessler tubes were found to be more satisfactory than a colorimeter or photometer for the extremely small amounts of Cu being determined.

Results and Discussion

The findings of Barron *et al.* [1935] already mentioned form a suitable starting point for the examination of the enzymic oxidation of ascorbic acid, since complete control of the reaction can be accomplished by arranging conditions to exclude all oxidation except that by an enzyme.

Some experiments were first performed with concentrations of $CuSO_4$ as catalyst ranging from 0·000001 m*M* to 0·001 m*M* in each flask with 25 μ-mol. ascorbic acid at *p*H 6·0. The reaction rates (Table 1) are in fair agreement with those observed by Barron *et al.*

Table 1. *Oxidation of ascorbic acid with $CuSO_4$ as catalyst*

*p*H = 6·0; temp., 25°: 25 μ-mol. ascorbic acid substrate.

m*M* $CuSO_4$	O_2 consumption μl./hr.
0·000001	7
0·000005	39
0·00001	71
0·0001	221
0·001	709

In order to obtain preparations of cucumber oxidase which would be free from adventitious heavy metals, possible reaction intermediaries and other interfering bodies, the juice from cucumber tissue was dialysed for several days against Cu-free water until the dialysate was free from Cu. The activity of the juice decreased very slightly from day to day during dialysis, the original rate of O_2 uptake being about 1500 μl. per hr. and the final rate after dialysis for 6–7 days being approximately 1000 μl. per hr. Fig. 1 illustrates such an experiment. Catalytic oxidation by haemochromogens which possess, molecule for molecule, a fraction of the catalytic power of Cu salts, is excluded from these experiments since tests on the enzyme preparations for Fe with the dipyridyl reagent were either negative or so weakly positive that the amount of haemochromogen (assuming that all the Fe was present as such) would be insignificantly small.

All salts having been removed from the enzyme preparations along with quinonoid and phenolic materials and organic molecules of small dimensions, some 50–100 ml. of the preparation were measured out for incineration and the resulting ash tested for Cu by measuring the rate of oxidation of ascorbic acid catalysed by 1·00 ml. of the solution of the ash. As the results in Fig. 2 show, the

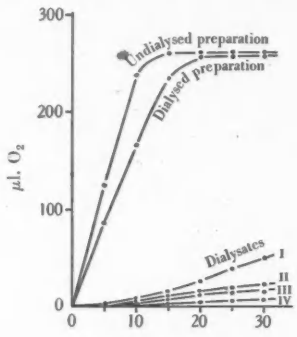

Fig. 1.

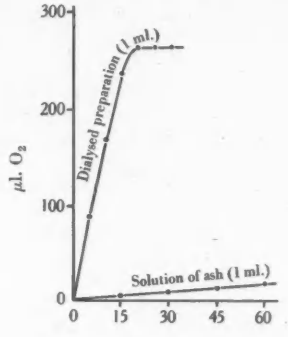

Fig. 2.

activity of 1 ml. of dialysed enzyme preparation is much greater than that of 1 ml. of the solution of the enzyme ash: that is, the activity of a given amount of enzyme preparation is much greater than the catalytic activity of the amount of Cu in that enzyme preparation. The results of further experiments illustrating this principle are given in Table 2.

Table 2

Exp.	Cu found in juice μ-mol./ml.	Ash activity μl. O_2 per hr.	Cu calculated from ash activity μ-mol./ml.	Enzyme activity μl. O_2 per hr.	μl. O_2 / hr. × μ-mol. Cu	μl. O_2 / min. × μg. Cu
17	0·0020	16	0·0022	1036	470,909	123
18	0·0020	20	0·0030	990	471,430	126
19	0·0021	102*	0·0200	1000	500,000	131
20	0·0020	16	0·0022	1044	474,545	124

Cu was determined colorimetrically and also by calculation from the catalytic activity of the ash preparations using the values in Table 1 as a basis for the calculation. The last two columns express the activities of the preparations (1) as rate of O_2 uptake per hr. per μmol. $CuSO_4$ and (2) as rate of O_2 uptake per min. per μg. Cu.

* For 10 ml. juice.

Since, according to the results of Barron *et al.* [1935], no other metal than Cu is likely to be involved in the catalytic oxidation of ascorbic acid and since, under the conditions which obtained in these experiments, possible interfering substances were removed by dialysis, it is necessary to postulate some complex of Cu to account for the difference between the activity of the dialysed enzyme preparations and the activity of the corresponding amounts of ash solutions. That the oxidation is primarily concerned with the amount of Cu present in the preparations is shown by the reasonably concordant figures which express the activities of different samples of dialysed enzyme preparations as the rate of O_2 consumption per μg. Cu (Table 2). The Cu complex postulated here is possibly

analogous to the Cu protein isolated by Kubowitz from potato. The dialysed enzyme preparations, however, clearly do not contain a polyphenol oxidase or a tyrosinase since experiments with *p*-cresol and catechol as substrate were negative, the rate of O_2 consumption being of the order of 5–10 μl. per hr. (which is little more than the 'blank') compared with 1000 μl. per hr. with ascorbic acid as substrate. Their behaviour and relatively constant copper : activity ratio suggest that there is present a discrete ascorbic acid oxidase.

Stotz *et al.* [1937] reported that the addition of cyanide and other substances forming insoluble complexes with Cu to cucumber juice inhibited the oxidase activity and that dialysis against water to remove the inhibitors restored the activity in every case except that in which cyanide had been added. This has been confirmed. In addition, experiments have been carried out with dialysed enzyme preparations to which NaCN and HCl were added to give a final concentration of *M*/100 in each case: the preparations with NaCN or HCl were dialysed separately against Cu-free water until the dialysates were free from cyanide or HCl. This required 2–3 days' dialysis, during which time the dialysates gave positive reactions when tested for Cu with sodium diethyldithiocarbamate. A further removal of Cu by dialysis against cyanide or HCl had therefore occurred, although Cu was no longer removable by simple dialysis against water. The enzyme preparations, after this further removal of Cu, were quite inactive in catalysing the oxidation of ascorbic acid.

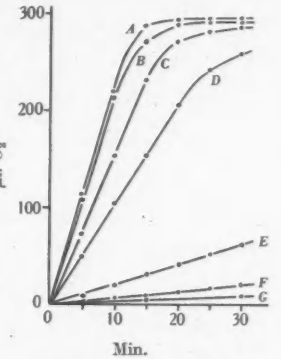

Fig. 3. *A*, undialysed preparation (1 ml.). *B*, dialysed preparation (1 ml.). *C*, restored preparation (10 × excess Cu). *D*, restored preparation (3 × excess Cu). *E*, 0·00002 m*M* $CuSO_4$. *F*, 0·000006 m*M* $CuSO_4$. *G*, ash solution (1 ml.).

It remained to restore the activity of the dialysed preparations. Samples of dialysed cucumber juice of known Cu content were dialysed with *M*/100 NaCN and *M*/100 HCl and then with Cu-free water until all cyanide or HCl had been removed (4–5 days). Three and ten times the calculated amounts of Cu were added as $CuSO_4$ to samples of the inactivated preparations and allowed to stand for a few hours at room temperature: the preparations were then dialysed against water for 24 hr. to remove any excess Cu.

Table 3

	O_2 consumption µl. per hr.
1 ml. dialysed preparation A	1248
1 ml. inactivated preparation B	0
1 ml. inactivated preparation + 0·00002 m*M* $CuSO_4$ C	834
1 ml. inactivated preparation + 0·000006 m*M* $CuSO_4$ D	558

Cu content of A. 0·0025 μ-mol./ml.
Cu content of A. 0·0022 μ-mol./ml. calculated from ash activity.
Cu content of C. 0·0030 μ-mol./ml.

The preparations inactivated with HCl remained inactive: this was probably due to the extensive denaturation and precipitation of protein which occurred during the inactivation period.

The preparations inactivated with NaCN were restored to activity and the results are shown in Table 3. The experiment is also recorded graphically in

Fig. 3, the curve for the O_2 uptake of the inactivated preparation being omitted as it was nil. The activity of the enzyme preparation was successfully restored to the extent of 67 and 45% by the addition of ten-fold and three-fold excesses of an inorganic Cu salt. Taken in conjunction with the inactivation of cucumber juice by the removal of Cu, with the relation between the oxidase activity and the Cu content of the dialysed preparations, and with the difference between the activities of Cu in the preparations and Cu in the ionic form, these facts provide evidence that there exists in autolysed cucumber juice a discrete ascorbic acid oxidase, and that the enzyme is a metal-protein in which the prosthetic group is Cu.

Summary

The ascorbic acid oxidase activity of cucumber juice is proportional to the amount of Cu in the juice after all 'free' Cu and other extraneous materials have been removed by prolonged dialysis against water. This activity is much greater than the catalytic activity of ionic Cu or Cu in the form of a loose non-specific combination with protein.

Complete removal of Cu inactivates the cucumber juice.

Dialysed cucumber juice inactivated by further dialysis with NaCN has been restored to activity by the addition of excess inorganic Cu salt; the restored preparation was more active in the oxidation of ascorbic acid than the amount of Cu salt used for reactivation.

It is concluded that cucumber juice contains a discrete ascorbic acid oxidase, a metal protein complex in which the prosthetic group is Cu, and which is not identical with polyphenol oxidase.

The authors wish to acknowledge a grant from the Earl of Moray Endowment Fund of this University which partly covered the expenses of this work.

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81. THE DETERMINATION OF VITAMIN C IN URINE

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(*Received 6 June 1941*)

The estimation of vitamin C in urine by direct titration, even after treatment of the urine to remove certain interfering substances, presents a number of difficulties. Direct titration in acid solution with 2:6-dichlorophenolindophenol (hereinafter called indophenol) determines the amount of ascorbic acid present but does not measure dehydroascorbic acid or any non-reducing complex of ascorbic acid. Since dehydroascorbic acid is as active physiologically as ascorbic acid [Borsook *et al.* 1937], this point is of some importance in view of the ease with which ascorbic acid is oxidized by air and other reagents even in acid solution when traces of copper are present. A further complication is introduced by the fact that at hydrogen ion concentrations less than *p*H 4·5 dehydroascorbic acid undergoes a non-oxidative irreversible change [Ball, 1937] and cannot be reconverted into ascorbic acid by ordinary methods. The total amount of vitamin C, therefore, cannot easily be determined by direct titration with indophenol. Indophenol, in acid solution, oxidizes not only ascorbic acid but many other substances which may and do occur in urine. The method of direct titration cannot be considered specific and is of little use in the examination of urine from scorbutic subjects and cases of latent scurvy in which the total amount of vitamin C excreted may be very small compared with the total amount of indophenol-reducing material in the urine.

In this connexion we have examined the method of Scarborough & Stewart [1937] for the determination of vitamin C in urine and have introduced certain modifications; a new method has also been developed in which the reduction of dehydroascorbic acid and the hydrolysis of non-reducing ascorbic acid complexes are accomplished by means of a dilute solution of $SnCl_2$ in dilute HCl. In both methods the true vitamin C, which is all present finally as ascorbic acid, is determined by an enzymic procedure.

Experimental

Treatment of urine

The urine used in the experiments was a freshly passed or a 24-hr. specimen, according to the nature of the experiment, and, unless otherwise stated, was normal. It was immediately acidified with glacial acetic acid, 10 ml. being added to each 100 ml. urine. It was found that the loss of dehydroascorbic acid, which is of considerable magnitude in urine and which occurs to a lesser extent in acidified urine, could be prevented by addition to the collecting bottle of 50 ml. of the $SnCl_2$ solution described below; this procedure in no way interfered with the subsequent analysis.

Removal of interfering substances by 'clearing'

Two routine methods were used. The first was that of Scarborough & Stewart [1937] in which equal volumes of urine, 20% mercuric acetate in 10% acetic acid and 10% acetic acid were mixed and centrifuged quickly (3 min.) and the supernatant liquid was saturated with H_2S until precipitation was complete. The black precipitate was filtered off and the filtrate freed from H_2S by bubbling through it a stream of wet CO_2 (10 min.).

The second clearing method was used after reduction by $SnCl_2$ when the urine filtrate was coloured and likely to contain sulphydryl compounds etc. A measured and usually small volume of 20% mercuric acetate in 10% acetic acid was added to the urine filtrate and the whole quickly saturated with H_2S. The precipitated sulphides were filtered off and the filtrate aerated as before. When complete clearing was unnecessary the tin was precipitated alone as the sulphide and the filtrate aerated.

Reduction of dehydroascorbic acid

Reduction of dehydroascorbic acid was carried out according to the method of Scarborough & Stewart [1937] by saturation with H_2S for 24 hr. The solution was freed from H_2S by aeration with wet CO_2 (30 min.).

$SnCl_2$ was also used to reduce dehydroascorbic acid and the solution which was found to be most convenient in practice was $M/10$ $SnCl_2$, $2H_2O$ in $M/4$ HCl solution. It was prepared by dissolving 22·25 g. $SnCl_2$ crystals (Analar) in 25 ml. conc. HCl with heat and diluting the solution to 1 l. This stock solution kept well on the addition of a small piece of granulated tin. 5 ml. were used to reduce a filtrate containing not more than 100 mg. dehydroascorbic acid.

The $SnCl_2$ solution made by B.D.H. Ltd. for arsenic tests is also a very useful stock solution: 0·20 ml. were sufficient to reduce a filtrate containing not more than 100 mg. dehydroascorbic acid. It is of particular value in the micro-method described below since excessive dilution is avoided.

Hydrolysis

The urine, or urine filtrate, after clearing, was refluxed for a measured time under an inert atmosphere and in acid solution. The acetic acid added to the urine made it sufficiently acid. Correction to the volume was applied in every case to allow for any slight loss of fluid while refluxing.

Enzyme oxidation

The acid filtrates obtained after hydrolysis and reduction were buffered with 25% NaOH to *p*H 6·0 using bromocresol purple as an external indicator; the volume of NaOH was measured from a burette. The enzyme solution was prepared by autolysis of cucumber tissue and the juice was used without further treatment after being tested for activity. 1 ml. of this juice was added to approximately 20 ml. of the buffered filtrate and the mixture aerated in the blood urea apparatus manufactured by Quickfit and Quartz, Ltd.; the aeration was stopped after 30 min. and the enzyme action brought to an end by the addition of 2 ml. 50% metaphosphoric acid. The solution was titrated after the protein precipitate had been removed by centrifuging.

Reductic acid was prepared according to the method of Reichstein *et al.* [1933]; it had M.P. 210° (uncorr.); quoted, 213°.

Reductone was prepared by the method of Euler & Martius [1933]. The material was not isolated but solutions were prepared freshly before use.

Discussion and results

The method of Scarborough & Stewart [1937] was investigated with regard to the hydrolysis of non-reducing complexes of ascorbic acid which they accomplished by refluxing the urine filtrate under an inert atmosphere for 24 hr.

Samples of urine were reduced with H_2S after clearing and weighed amounts of ascorbic acid added to aliquots of these to study the effect of hydrolysis at

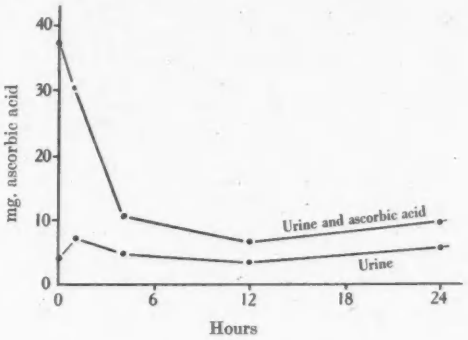

Fig. 1.

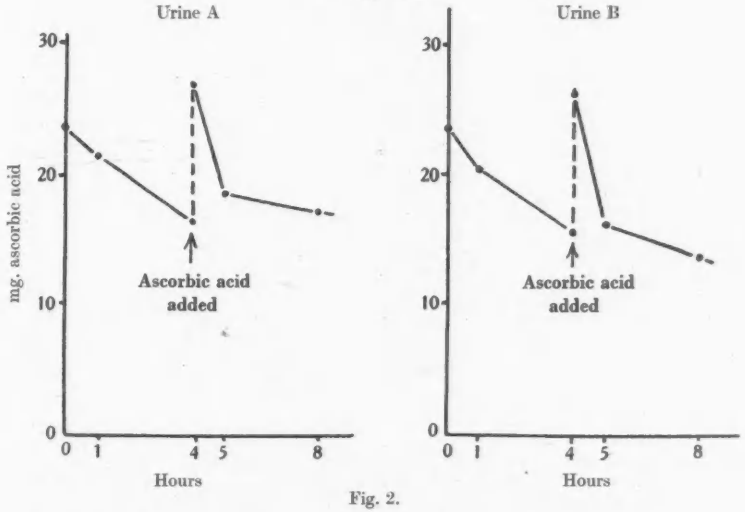

Fig. 2.

higher concentrations of ascorbic acid than those in the urine filtrates. The samples were submitted to hydrolysis and small measured volumes were withdrawn at timed intervals for reduction, clearing and titration. As is evident from Fig. 1, a slight increase in the indophenol-reducing power after hydrolysis for 24 hr. need not be due to ascorbic acid being liberated from non-reducing complexes, since it is obvious that the acid is destroyed during the operation.

That this destruction is not a stoichiometric reaction with some other substance in the urine filtrate is shown by the following experiment. Urine samples were cleared and reduced as before and ascorbic acid was added. After hydrolysis for 4 hr., sufficient ascorbic acid was added to bring the concentration up to the original level and the hydrolysis was allowed to proceed; samples were withdrawn for reduction, clearing and titration and the results are expressed graphically in Fig. 2. The rapid rate of destruction during the second period of hydrolysis at a rate comparable with that during the first period of 4 hr. suggests a catalytic reaction rather than a stoichiometric reaction with some other substance in solution. The disappearance of ascorbic acid, presumably oxidative since it was measured by the decrease in the indophenol-reducing power, is rather difficult to explain in view of the anaerobic conditions prevailing, but it is quite real and non-reversible by reduction with H_2S. The same phenomenon was observed when mineral acid was used instead of acetic acid to acidify the urine, so that the hydrogen ion concentration was well within the range in which dehydroascorbic acid is stable if this substance were formed by some obscure oxidation process.

The graphs in Fig. 1 show that the main part of the hydrolysis which liberated indophenol-reducing material occurred within the first hour, so that the long 24-hr. period of hydrolysis previously included in the method of Scarborough & Stewart was not necessary. The total indophenol-reducing power was measured by prolonged treatment with H_2S which, as shown by Scarborough & Stewart, has a hydrolytic action as well as a reducing action.

It is also evident that with a suitable reducing agent for the rapid reduction of dehydroascorbic acid to ascorbic acid, the time required for a single estimation could be decreased considerably. Some inorganic reagents were investigated such as titanous and stannous chlorides but only $SnCl_2$ in very dilute HCl solution appeared to reduce solutions of dehydroascorbic acid. Since the tin salt could be removed either by clearing with mercuric acetate or by direct precipitation as the sulphide, the reagent was further investigated and the solution most suitable for the purpose was found to be $M/4$ HCl solution containing $M/10$ $SnCl_2$, $2H_2O$. The recoveries of ascorbic acid from solutions of dehydroascorbic acid by treatment with $SnCl_2$ solution in the cold for 10 min. are shown in Table 1.

Table 1. *The dehydroascorbic acid solutions were prepared by shaking up with charcoal standard solutions of ascorbic acid and removing the charcoal by filtration*

Ascorbic acid, mg./5 ml. solution	Ascorbic acid, mg./5 ml. solution after oxidation	Recovery by $SnCl_2$, mg.	Recovery %
0·050	0·000	0·050	100
0·086	0·001	0·087	101
0·102	0·000	0·100	98
0·490	0·005	0·491	101
0·671	0·001	0·670	100
0·880	0·002	0·878	99

Since the determination of ascorbic acid appeared to require both hydrolysis and reduction, operations which, under the acid conditions prevailing, would be difficult to separate, both procedures were combined in one operation so that the efficacy of $SnCl_2$ could be compared with that of H_2S. Preliminary experiments had shown that ascorbic acid was quantitatively recoverable after refluxing for 4 hr. with $SnCl_2$ solution under an inert atmosphere (Table 2), so an attempt was

made to hydrolyse and reduce the urine filtrates by refluxing them with $SnCl_2$ solution after clearing. The results of this experiment are shown in Fig. 3 along with the total indophenol-reducing power determined by the method using H_2S. The reducing power reached a maximum after hydrolysis for 60–90 min. on a boiling water bath, and this maximum was the same as that reached when

Table 2. *Recovery of ascorbic acid after refluxing with $SnCl_2$ solution on the hot water bath for 4 hr. under an inert atmosphere*

Ascorbic acid, mg.	Solvent, 50 ml.	Ascorbic acid recovered, mg.	Recovery %
12·3	*N*/100 HCl	11·9	97
10·5	"	10·2	97
8·23	5% HAc	8·22	100
21·0	"	21·6	103
5·50	1·5% HPO_3	5·50	100
4·27	"	4·27	100
21·0	10% HAc	20·5	98
12·5	"	12·5	100

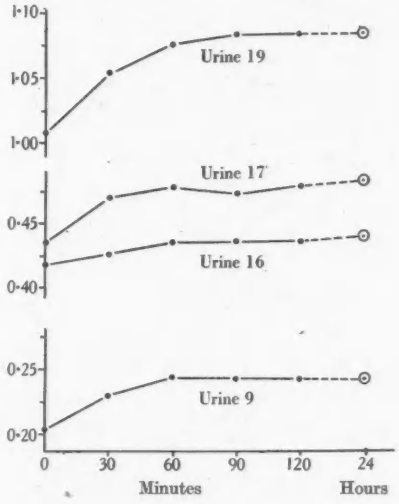

Fig. 3.

using H_2S. The 90 min. interval was used for routine purposes and some comparative results for the two methods are shown in Table 3. It is interesting to note that the same total indophenol-reducing power may be reached in a variety of ways: refluxing on a water bath with $SnCl_2$ under N_2 for 90 min., treatment with $SnCl_2$ solution in the cold for 24 hr., and prolonged treatment with H_2S all give the same maximum indophenol-reducing power with a particular sample of urine. Treatment with $SnCl_2$ in the cold for 10 min. reduces only the dehydroascorbic acid in the urine. In Table 4 are collected the results of several analyses of urines by these different methods. It was found that simple hydrolysis followed by reduction rarely produced the same maximum as the other methods, but simultaneous reduction and hydrolysis proceeded satisfactorily.

Table 3. *Comparison of indophenol-reducing powers of various urines after reduction by $SnCl_2$ or H_2S*

No. of urine	$SnCl_2$ ascorbic acid m*M*/l.	H_2S ascorbic acid m*M*/l.
6	0·43	0·42
7	0·37	0·36
17	0·30	0·30
19	0·210	0·205
27	0·059	0·059
28	0·074	0·077
29	0·055	0·053

The last three urines were from a scorbutic subject.

Table 4. *Total indophenol-reducing power of 4 urines determined by different methods and amount of increase by simple reduction (10 min.)*

Figures represent m*M*/l. ascorbic acid

No. of urine	21	19	17	10
Cleared only	0·419	0·241	0·188	0·054
$SnCl_2$ 10 min. (cold)	0·493	0·316	0·218	0·055
$SnCl_2$ 90 min. (90°)	0·550	0·295	0·259	0·146
$SnCl_2$ 24 hr. (cold)	0·548	0·298	0·257	0·145
H_2S method	0·551	0·296	0·257	0·146

The suggestion that ascorbic acid is present in urine partly in a non-reducing combined form is borne out by the results quoted above in which hydrolysis is necessary to produce the maximum reducing power of the urine filtrates, although treatment with $SnCl_2$ for 10 min. in the cold is sufficient to reduce dehydroascorbic acid in pure solution. Experiments with enzymic oxidation described below show that part of the increase in reducing power is due to ascorbic acid.

As a final and necessary test of the $SnCl_2$ method the true ascorbic acid was determined by enzymic oxidation and compared with the values obtained with the H_2S method. The results are shown in Table 5 and the agreement between the two methods is satisfactory.

Table 5. *The urines examined here are from a scorbutic subject before and after treatment with vitamin C. The figures represent the reducing power of the filtrates expressed as* m*M*/*l. ascorbic acid*

No. of urine	Total indophenol-reducing power		Reducing power after oxidation (non-vitamin material)		Difference, vitamin C	
	$SnCl_2$	H_2S	$SnCl_2$	H_2S	$SnCl_2$	H_2S
1_1	0·149	0·147	0·060	0·059	0·089	0·088
4_1	0·157	0·152	0·077	0·071	0·080	0·081
11_1	0·041	0·039	0·043	0·040	—	—
16_1	0·053	0·052	0·035	0·034	0·018	0·016
15_2	0·904	0·898	0·040	0·040	0·864	0·858
16_2	0·381	0·378	0·045	0·042	0·336	0·336
17_2	0·925	0·925	0·067	0·069	0·858	0·856

Specificity of the enzyme under the conditions of analysis

The specificity of the methods for the determination of ascorbic acid discussed above depend to a great extent upon the specificity of the cucumber oxidase used in the final operation. Zilva & Snow [1938] have shown that the

enzyme is not specific for ascorbic acid and that it oxidizes many other compounds of the same dienol type. Nevertheless, it seemed that the inclusion of the enzymic oxidation in the analytical procedure was desirable since, under the conditions of analysis, ascorbic acid is oxidized rapidly and completely, whereas other dienol compounds which have been examined are oxidized slowly and incompletely. There is as yet no evidence that these compounds are present in normal or scorbutic urines and, in addition, the enzymic oxidation excludes from the final result some indophenol-reducing material which is not oxidized by cucumber oxidase.

The experiments on the specificity of the enzyme were carried out in exactly the same way as the enzymic oxidations in the analytical procedure, using reductic acid and reductone solutions instead of urine filtrates. Control tubes containing known amounts of ascorbic acid were set up each time and it was found that the acid was oxidized completely in every case. The *p*H of the solutions was checked, before and after oxidation, in a Beckmann *p*H meter. The results are shown in Tables 6 and 7. The rates of oxidation of reductic acid and

Table 6. *The figures represent m*M *reductic acid in 20 ml. solution, correction being applied for the dilutions involved*

Reductic acid solution	Aerated at *p*H 6·0 + enzyme	Aerated at *p*H 6·0
0·270	0·160	0·179
0·164	0·083	0·083
0·083	0·025	0·024

The *p*H was checked before and after aeration in a Beckmann *p*H meter.

Table 7. *The figures represent m*M *reductone/20 ml. solution, correction being applied for the dilutions involved*

Reductone solution	Aerated at *p*H 6·0 + enzyme	Aerated at *p*H 6·0
0·375	0·148	0·182
0·624	0·431	0·432
0·740	0·446	0·456
0·787	0·313	0·388
0·948	0·406	0·415
1·301	0·662	0·762

The *p*H was checked before and after aeration in a Beckmann *p*H meter.

reductone were not significantly greater in presence of the enzyme than in its absence; under the conditions of analysis there was probably a fair amount of catalytic oxidation by copper as no special precautions were taken to exclude this metal. Thus, although it cannot be claimed that the procedure is completely specific, interference is limited to a few compounds of the same dienol type as ascorbic acid which are partly oxidized in the last operation and whose presence in normal or scorbutic urines is not yet conclusively proved.

Routine procedure for the determination of vitamin C in urine

Reagents. Glacial acetic acid and 10% acetic acid.
20% mercuric acetate in 10% acetic acid.
M/10 $SnCl_2$ in *M*/4 HCl (or B.D.H. $SnCl_2$ for arsenic tests).
H_2S gas (from Kipp apparatus), CO_2 and N_2.
Indophenol solution equivalent to 5 mg. ascorbic acid per 100 ml.
25% NaOH.
50% metaphosphoric acid, freshly prepared.
Autolysed cucumber juice.

Macro-method

The urine, acidified with glacial acetic acid to a concentration of 10% acetic acid, need not be cleared unless it has a high specific gravity, is darkly coloured and is opaque and likely to contain considerable quantities of interfering substances as in the case of urines containing large amounts of sulphur.

To 50 ml. of urine or urine filtrate after clearing, add 10 ml. $SnCl_2$ solution or 0·50 ml. B.D.H. reagent and reflux under N_2 on a boiling water bath for 90 min. Cool quickly, add 20 ml. mercuric acetate solution, pass H_2S gas quickly through the solution until precipitation is complete and filter off the precipitate. Set aside 15–20 ml. for titration after removal of H_2S by aeration with CO_2 (10 min.) and buffer a 20 ml. portion with 25% NaOH to *p*H 6·0, measuring the alkali from a burette. Add 1 ml. of autolysed cucumber juice and aerate in a blood urea apparatus for 30 min. Stop the aeration and add 2 ml. 50% metaphosphoric acid solution, centrifuge and titrate an aliquot of the supernatant liquid. The difference between the results of the two titres, expressed as ascorbic acid and with due allowance for the dilutions involved, represents vitamin C.

Tables 8 and 9 contain results of analyses on urine and food samples carried out by this method.

Table 8. *Some analyses of scorbutic urines carried out by the $SnCl_2$ method*

Ascorbic acid is expressed as mg./100 ml. urine and the non-vitamin reducing material is also expressed as mg. ascorbic acid/100 ml. urine.

No. of urine	Day	Total indophenol-reducing power	Non-vitamin reducing material	Ascorbic acid	Daily excretion mg.
1	3	1·36	1·07	0·29	
1	5	1·14	0·86	0·28	
1	10	1·80	0·73	1·07	
1	12	7·50	0·70	6·80	
1	15	15·95	0·70	15·25	
1	16	6·70	0·79	5·91	
1	17	16·30	1·15	15·15	
2	6	1·23	0·62	0·61	5·96
2	7	0·53	0·61	—	—
2	8	2·50	0·73	1·77	16·6
2	9	2·27	1·28	0·99	7·1
2	14	1·04	0·72	0·32	5·7
2	16	0·87	0·63	0·24	4·2
2	17	0·83	0·59	0·24	4·0
2	19	0·71	0·62	0·09	1·3
2	20	0·86	0·61	0·25	2·9
3	11	0·75	0·65	0·10	1·4
3	14	1·01	0·68	0·33	4·7
3	18	0·50	0·53	—	—
3	19	0·78	0·68	0·10	1·4
3	20	2·17	0·61	1·56	27·0
3	21	6·00	0·76	5·24	86·4
3	22	5·31	0·68	4·63	51·4
3	23	1·86	0·72	1·14	11·4

Ur 1 and Ur 3 are urines from a scorbutic subject during treatment with vitamin C; Ur 2 is a urine from a scorbutic subject before treatment with vitamin C.

Table 9. *Analyses of dried vegetables*

The vegetables were extracted for a prolonged period with 5% metaphosphoric acid and the extract analysed by the $SnCl_2$ method. Owing, probably, to the drying process, the extraction was very easy to carry out, the solutions being coloured but free from excess of protein material.

Vegetable	Ascorbic acid mg./100 g.
Julienne	2·6
French beans	11·2
Turnips	10·8
Spinach	3·5
Cabbage	26·5
Onions	7·0

The values found in most cases are much the same as those quoted for cooked vegetables still retaining their tissue water but separated from the cooking water. The low value for spinach is interesting in view of the relatively high heavy-metal content of this vegetable.

Micro-method

The procedure in principle is exactly the same: smaller quantities are used and a micro-diffusion burette is used for the titration.

20 ml. acidified urine or urine filtrate are refluxed with 5 ml. $SnCl_2$ solution or 0·20 ml. B.D.H. reagent in a micro-reflux apparatus, with provision for a stream of N_2 to pass through the liquid, for 90 min. A 20 ml. portion of the mixture is cleared with 10 ml. mercuric acetate and the final filtrate is titrated in 1 or 2 ml. portions. Half quantities are used for the enzymic oxidation and a 2 ml. portion is taken for titration.

SUMMARY

The method of Scarborough & Stewart [1937] for the determination of vitamin C in urine has been investigated and certain modifications have been introduced.

A new method for the determination using $SnCl_2$ in dilute HCl solution for the reduction and hydrolysis of urine has been developed. In both methods, the true vitamin C was determined by an enzymic method.

The specificity of cucumber oxidase under the conditions of analysis has been investigated and the results uphold the utility of the enzymic procedure.

Ascorbic acid and other indophenol-reducing substances are liberated during the combined reduction and hydrolysis of urine.

Some analyses are reported. Scorbutic urines show very low concentrations of vitamin C and occasionally none is detectable.

Dried vegetables contain amounts of vitamin C of roughly the same order as the amounts in cooked vegetables retaining their tissue water but without the cooking water.

The authors are grateful to the Earl of Moray Endowment Fund of this University for a grant which partly covered the expenses of this work.

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82. A METHOD FOR ESTIMATING THE POTENCY OF HEPARIN PREPARATIONS

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(*Received* 6 *June 1941*)

THE earliest, crude preparations of heparin, made by the method of Howell & Holt [1918], were labelled in terms of a purely biological unit, defined as the minimum quantity of the material necessary to keep 1 ml. of cat's blood fluid for 24 hr. at 0°. The disadvantages of units so defined, in terms of some potentially variable animal reaction, are now generally recognized. Activity ought rather to be stated in terms of a standard preparation, a unit being then the specific activity contained in a fixed weight of a standard preparation, and measurable by any comparative biological test which is quantitative and specific. The crystalline barium salt of heparin [Charles & Scott, 1936] would appear to furnish a suitable standard of this kind, and Murray & Best [1938] have already proposed the use of a heparin unit, defined as the activity present in 0·01 mg. of this material. Before such a standard and unit can be recommended, however, for general adoption, there are several points concerning this barium salt on which further information is required.

(1) It is important to know whether the crystalline barium salt can be regarded as a pure and accurately reproducible chemical entity, so that all samples of it, complying with certain physical and chemical criteria of purity, can be assumed to be identical in the specific heparin activity. Even if the evidence were to show that different samples of the salt, indistinguishable by ordinary physical and chemical tests, nevertheless vary in activity, this would not, by itself, condemn the barium salt as an unsuitable material for a standard. It would, however, necessitate the definition of the standard, not simply as any pure sample of the barium salt complying with certain criteria, but as a particular quantity of the barium salt, kept in a recognized control laboratory under conditions satisfactorily guaranteeing the complete stability of its activity. Similarly, the unit would then have to be defined as the specific activity contained in a certain weight, not of the crystalline barium salt, wherever prepared, but of this particular standard sample of it.

(2) In any case, if the barium salt is to be regarded as suitable for adoption as a standard material, on any basis, it is important to know whether conditions can be found which will ensure the permanent stability of its specific heparin-activity; and, if so, whether these conditions are compatible with the convenient issue of the samples of the standard, and with its use by those requiring it as a basis of practical biological assay.

(3) A material standard should, ideally, furnish a basis for the definition of the unit applicable in any comparative biological test, and therefore independent of the details of the test so used, provided only that this measures the specific activity in question with sufficient accuracy.

My purpose in the present paper is to describe a method which gives results of good reproducibility, is very economical in time and animal material and

requires no special skill or judgement, since its end-point is sharp and objective. On the other hand, it reveals differences in activity between different samples of heparin, which are definitely greater than those determined for the same samples by methods using whole mammalian blood. The contrast between the results obtained by the two different types of method is sufficient to suggest that even the crystalline barium heparin contains more than one active material; that the activities of different components impede different factors concerned in the complex process of the clotting of shed blood; and that the component which is measured in the test using the clotting of recalcified plasma, as here described, is less stable than another component or components, the measurement of which also enters into a test using the clotting of whole blood.

Principle

Oxalated horse plasma, to which heparin has been added, is recalcified in the presence of an excess of thrombokinase. The clotting time depends on the concentration of heparin. A simple apparatus facilitates the simultaneous measurement of the clotting time in several vessels, containing serial concentrations of the unknown and of the standard heparin respectively.

Reagents

(1) *Oxalated horse plasma.* 9 vol. of blood are run from the jugular vein into a chilled paraffined vessel containing 1 vol. of 0·1 *M* potassium oxalate. Prompt and thorough mixing is essential. The oxalated blood is kept on ice until the plasma can be removed by centrifuging for 20 min. at 2000 r.p.m. The separated plasma is stored at 2°. Its clotting time when recalcified increases with storage, and plasma more than a week old is unsatisfactory for the assay: at room temperature it deteriorates still more rapidly. If, however, the oxalated horse plasma is completely dried in the frozen state while it is still fresh, and then stored under dry nitrogen in the cold, it retains its clotting properties unchanged for an indefinite period. The use of such a dried preparation greatly increases the convenience of the method. The bulked plasma is best distributed into 1 oz. bottles, each containing 10 ml., and kept frozen until drying can be commenced: the contents of one bottle suffice for a day's assays. The reconstituted plasma deteriorates at the same rate as fresh plasma: 4 hr. at 20° cause a measurable loss of clotting activity, which is, however, too small to affect the accuracy of the assay; any longer exposure at room temperature should be avoided.

Human, rabbit or cat plasma gives less satisfactory results, the end-point being less sharp in any of these cases than that obtainable with horse plasma.

(2) *Heparin* is dissolved in 0·9% NaCl. The concentration should be that which produces a clotting time of between 70 and 500 sec. in the recalcified system. With the use of fresh oxalated plasma this will be approximately 2 to 4 Toronto units per ml. It is convenient to use 3 concentrations each of standard and unknown: these may be in the ratio 80 : 100 : 125.

(3) *0·025*M $CaCl_2$.

(4) *Thrombokinase solution.* A rabbit is anaesthetized with nembutal or dial and bled out from the abdominal aorta under artificial ventilation. The right ventricle is incised, and the fore parts are perfused from the thoracic aorta with Locke's solution until the fluid returning by the jugular vein is colourless. The brain is then removed, freed from dura and ground in a mortar to a smooth paste. This is spread in a thin layer on a glass plate and dried, first in air at 37°,

and then *in vacuo* over P_2O_5. The powdered material can be kept for several months in the refrigerator without appreciable loss of potency. On the day of the experiment, a 3% suspension is made in 0·9% NaCl. This is warmed to 55° for 10 min. in a water bath, with thorough stirring; solid particles are then removed by centrifuging for 10 min. at 2000 r.p.m., and the milky supernatant is used. More prolonged centrifuging gives a clearer but less active fluid.

Glass-distilled water is used for all solutions, and all glassware must be thoroughly clean.

Procedure

Double-bulbed pyrex clotting tubes of the form shown in Fig. 1 are used. The design is of some importance: the bulbs should be spherical rather than tubular in shape, and the angle between them should be great enough to permit the contents of one bulb to drain completely into the other when the tube is inclined through 70° from the vertical. Six such tubes are conveniently held by spring clips on a horizontal wooden rack affixed to a rod which can be tilted by hand about its long axis. The whole apparatus is so arranged that it can be placed in a thermostat at 37°.

Fig. 1. Half actual size.

0·1 ml. of thrombokinase solution and 0·1 ml. of 0·025 *M* $CaCl_2$ are measured into one bulb of each tube, and 0·1 ml. of heparin solution and 0·1 ml. of oxalated plasma into the other bulb. A 0·5 ml. micrometer syringe of the type devised by Trevan [1926] is useful for measuring the reagents. Since heparin, added to plasma, does not immediately become fully effective [Quick, 1936], the plasma and heparin in each tube should be mixed at as nearly the same moment as possible. The rack carrying the tubes is placed in the thermostat, and left for 20 min., to enable the effect of the heparin on the plasma prothrombin to reach a practically uniform degree in all tubes. The reagents are then mixed simultaneously in all tubes by tilting the rack several times. The end-point is the formation of a solid clot, which is not displaced when the tube is tilted, and the time required is noted for each tube. It is important not to tilt the rack too often or too far, since the incipient clot may thereby be dislodged or broken, and the apparent clotting time prolonged. A horizontal mark, on each bulb of the series in which the clotting mixtures are kept for observation, facilitates the determination of the end-point.

The clotting time can, with a little practice, be measured with an accuracy of 5–10%. Since an increase of 25% in the heparin concentration may, over the optimal range, more than double the clotting time, the precision of the method is potentially high, and, indeed, is limited in practice by other errors than those involved in judging the end-point.

Calculation

If clotting occurs at the same time in two tubes, these are judged to contain the same number of anticoagulant units. Intermediate values are obtained by plotting the clotting time against concentration of the standard heparin, and interpolating for the clotting time of the unknown. No simple formula expresses the relationship between clotting time and heparin concentration: this varies somewhat from experiment to experiment, unless elaborate precautions are taken to maintain constant activity of the reagents.

A specimen protocol shows the type of result obtained.

Heparin	Concentration (μg./ml.)	Clotting time (sec.)
Standard	20	110
,,	25	230
,,	31·2	500
Unknown	64	100
,,	80	190
,,	100	420

Whence 80 μg. of unknown = 23·2 μg. of standard; 100 μg. of unknown = 29 μg. of standard; and potency of unknown = 29% potency of standard.

Error of the method

Five dilutions of a standard heparin solution, the strengths of which were unknown to the experimenter, were assayed in triplicate, 3 tubes of standard and 3 tubes of unknown being used in each experiment. The standard deviation from theory of the 15 single assays was 2·9%, the largest error being 5·6%. The standard deviation of the 5 triplicate assays was 1·1%, the largest error being 1·5%.

The result given by the method is unchanged by the addition, to one part by weight of the sample to be assayed, of two parts of any of the following substances: dried rabbit's brain, Ba (as $BaCl_2$), NaOH, HCl, tricresol, mucoitin sulphuric acid, heparin inactivated by prolonged boiling with acid alcohol. The method can therefore be used for the assay of preparations of the barium salt of heparin, or of solutions for clinical use containing tricresol as preservative, and, presumably, for following the activity of crude material during its purification.

Comparison with other methods

While this method was being developed, it was observed, unexpectedly, that certain highly purified preparations of heparin showed differences in activity of as much as 35%, which could not be explained by differences in ash or water content. Such variations in potency were found among samples of the crystalline barium salt, as well as among samples of the sodium salt prepared from it, and were perfectly reproducible from one assay to another. It was therefore important to know whether such qualitative differences would lead to the assignment of different relative potencies for these preparations, when they were assayed by other methods. I was fortunate in obtaining the co-operation of Dr F. J. Dyer and Dr R. Wien of the Department of Pharmacology of the College of the Pharmaceutical Society, and of Mr W. A. Broom and Mr E. M. Bavin of the Pharmacological Department of Boots Ltd., who had been using for the assay of heparin, in their respective laboratories, methods based on the use of fresh rabbit's blood. They will themselves report in more detail some of their results, but they permit me to summarize them here. Briefly, where unexplained differences of potency were found by the present method, they were also found by the rabbit's blood assay, but were smaller in the latter case: the difference between the results of the two methods was, in two cases out of five, statistically significant. One of these was a crystalline barium salt preparation which, compared with the temporary British Standard (110 units/mg.) assayed 73 units/mg. by the plasma-kinase method, and 92 units/mg. by the rabbit's blood method. The other was a specimen of the same barium salt, whose activity had been deliberately reduced by treatment with acid methyl alcohol: it assayed 19 units/mg. by the plasma-kinase method and 40 units/mg. by the rabbit's blood

method. From these last results it is clear that the partial inactivation caused by acid treatment appears greater by the plasma-kinase test than by the whole-blood test. It seems probable that most of the differences of potency found among samples of 'pure' heparin, and the divergent results obtained by different assay methods, may be due to the presence of varying amounts of material whose activity has thus been reduced by acid treatment. In one experiment, in fact, recrystallization of the barium salt in the presence of warm acetic acid, according to the usual technique, was found to reduce the potency by some 35% as found by the plasma-kinase test; on the other hand, recrystallization at nearly neutral reaction, in the presence of excess barium acetate, left the potency unchanged. If it is true that any submaximally active heparin preparation has been partly denatured by acid during the processes leading to its isolation, it follows that the plasma-kinase test gives a more delicate indication of the extent of the denaturation than does the whole-blood test. It does not follow, however, that the plasma-kinase test supplies more reliable data on the therapeutic efficiency of the material, for nothing is known about the relative activity of partially denatured heparin in the human circulation.

The present method is believed to offer several advantages over other biological methods on ground of convenience. By its use one worker can complete within a working day 8 or 10 comparisons, the results of which are reproducible within limits of $\pm 5\%$; no particular skill or judgement is required, and the cost in animal material is low, one rabbit's brain supplying enough thrombokinase for some 40 determinations. The question of the suitability of this method, as a measure of that action of heparin required in practical use, is considered further in the following paper.

The choice of a standard heparin

It has already been noted that Murray & Best [1938] have suggested that the crystalline barium salt would be a suitable material. It is clear, however, that samples of the barium salt, in spite of their crystalline nature and their apparently identical behaviour in physico-chemical tests, may vary considerably in anticoagulant potency, even when prepared from the same animal species. Recent work by Jaques & Waters [1940] and Jaques [1940] shows that still larger potency differences may be expected for crystalline preparations of barium-heparin from different animal species, again with no corresponding differences which can be detected by the physical and chemical methods so far applied. If the standard were to be made of the crystalline barium salt of heparin, it would, therefore, be necessary to choose a particular sample of the salt as the standard, and to define the unit of anticoagulant activity in terms of this sample. It must be remembered, however, that the chief advantage expected from using the barium salt as a standard depended on its supposed purity and invariability. If it is not, in fact, invariable in activity, we have to consider whether it is otherwise suitable.

On such other grounds it would seem preferable to use a sample of the sodium salt rather than the barium salt as a standard. The barium salt as crystallized contains some 12% of water and its aqueous solutions are strongly acid [Charles & Todd, 1940]. In view of the well-known susceptibility of heparin to acid inactivation, its stability over a long period might be doubted. I have, in fact, found that a sample of crystalline barium salt, which had been kept at laboratory temperature for about a year, had retained only about one-third of the potency expected for it. The possibility of a slow inactivation in the refrigerator, though a longer period might be required for its certain detection,

cannot be ruled out. The sodium salt, on the other hand, can be prepared in the fully neutralized condition, from material purified by crystallization as the barium salt. I have found it to be extremely stable, even in dilute solution and at high temperatures: unlike the barium salt, it can be sterilized by autoclaving without loss of potency. The standard sodium salt might be stored in the completely dried state in the central control laboratory, and (to avoid the difficulties of weighing small quantities of hygroscopic material) issued in the form of a sterile solution having a defined period of currency.

Summary

1. A method for the standardization of heparin preparations is described. It involves the use of oxalated horse plasma, which is recalcified in the presence of heparin and an excess of thrombokinase. It gives results reproducible to $\pm 5\%$.

2. Oxalated horse plasma, though inconveniently unstable in the fluid condition, appears to keep indefinitely when completely dried in the frozen state, and retains its clotting properties unchanged when redissolved.

3. The sodium salt of heparin, prepared from the crystalline barium salt, is probably more stable than the barium salt itself, and is therefore a more suitable material for use as a permanent reference standard.

4. The limitations of methods for the assay of heparin are discussed.

I wish to thank Sir Henry Dale for his interest and advice, and Prof. A. R. Todd for several samples of heparin. I am much indebted to Dr R. I. N. Greaves of Cambridge University for the preparation of a large stock of stable dried plasma.

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Addendum

Since this paper was written, Jaques & Charles [*Quart. J. Pharm. Pharmacol.* 1941, **14**, 1] have described, among other methods for the standardization of heparin, one which depends on its anticoagulant activity in a system consisting of thrombin and oxalated blood. They note two drawbacks of the method: (*a*) barium interferes by precipitating oxalate, so that samples of barium heparin cannot be accurately assayed; (*b*) the clotting properties of oxalated blood (and still more so those of oxalated plasma) change with keeping, so that reproducible results are secured with difficulty. The method which I have described in this paper, although it uses oxalated plasma, avoids these objections. The quantity of barium added in the form of heparin barium salt alters the Ca concentration in the clotting mixture by less than 2%, a change insufficient appreciably to affect the clotting time, as shown by my control experiments in which barium was deliberately added in the corresponding proportion; and the use of plasma stored in the stable dry form removes any uncertainties due to variations in the composition of this reagent.

83. A COLORIMETRIC METHOD FOR THE STANDARDIZATION OF HEPARIN PREPARATIONS

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(*Received 6 June 1941*)

It is a fact well known to histologists that certain basic dyes undergo a change of tint when taken up by particular tissue elements, especially cartilage, mucus and the granules of the so-called 'mast cells'. The chemistry of this phenomenon, named 'metachromasia' by Ehrlich, has been studied in some detail by Lison [1935]. This investigator concluded that the change of colour in the dye is due to the formation of the tautomeric imino-base, accompanied by an increased degree of molecular aggregation; and that all the substances which show the metachromatic staining contain organically combined sulphuric acid. Any sulphuric acid ester, in fact, provided that its molecular weight is not too low, will produce the characteristic colour change when added to an aqueous solution of a metachromatic dye. Now most of the substances known to be active in delaying the clotting of blood have large molecules containing esterified sulphuric acid; and, conversely, most substances having this structure appear to be anticoagulants [cf. Huggett & Rowe, 1933; Bergstrom, 1936; Chargaff *et al.* 1936]. Thus anticoagulant activity, and the capacity for giving the metachromatic reaction, are related to the same type of chemical structure. The effectiveness of any substance in producing the colour change appears, indeed, as will be shown, to be roughly proportional to its anticoagulant potency.

Heparin contains esterified sulphuric acid [Jorpes, 1935], and gives the metachromatic reaction with toluidine blue [Jorpes *et al.* 1937]. The reaction has been used with success as a histochemical test for heparin [Jorpes *et al.* 1937; Wilander, 1939]. It has not, however, been applied hitherto for the standardization of heparin preparations; and the purpose of this paper is to specify conditions under which such an application is possible. The dye which has been chosen is toluidine blue.

The reaction between toluidine blue and heparin

If a weak (e.g. 0·01 %) solution of heparin is added to an equal volume of an aqueous toluidine blue solution of the same strength, the tint of the dye solution changes immediately from blue to red-violet. With smaller amounts of heparin, intermediate shades of purple are produced. The absorption of such mixtures for light of a given colour can be measured in a suitable instrument, and is a function of the concentration of heparin and of the dye.

This simple procedure is not, by itself, very useful as a basis for the colorimetric estimation of heparin, since (*a*) the absorption spectra of the two forms of the dye overlap, and (*b*) the heparin-dye complex is precipitated at a rate depending on the concentration of the reagents and on other factors. If, however, the mixture is shaken with an immiscible organic solvent, the heparin-dye complex is removed by adsorption at the interface, while the uncombined dye

remains in the aqueous phase, and retains its normal tint. The proportion of dye removed can now be determined by measuring the absorption of the aqueous phase, the calculated loss then serving as a measure of the added heparin.

Fig. 1 shows the effect of adding different amounts of a heparin preparation to a standard solution of toluidine blue, the heparin-dye complex having been

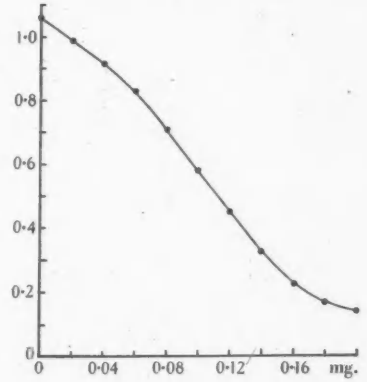

Fig. 1.

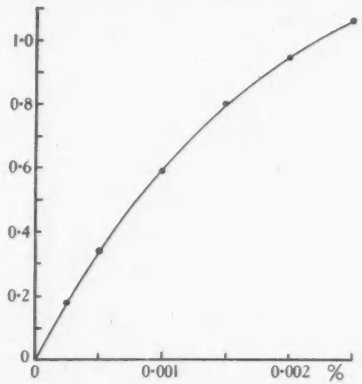

Fig. 2.

Fig. 1. Abscissae: mg. of heparin added to 10 ml. of 0·0025% toluidine blue solution. Ordinates: absorptiometer readings (orange filters) after extraction with light petroleum.

Fig. 2. Abscissae: concentration in % of toluidine blue in aqueous solution. Ordinates: absorptiometer readings.

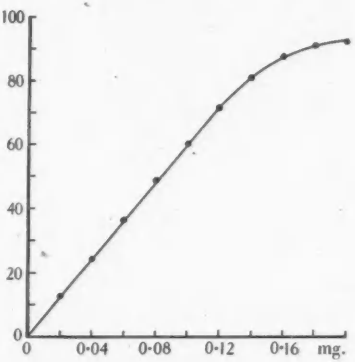

Fig. 3. Abscissae: mg. of heparin. Ordinates: % of dye removed from 10 ml. of 0·0025% toluidine blue solution. (From data of Figs. 1 and 2.)

removed by shaking with light petroleum. The extinction of the aqueous phase was measured in a Hilger 'Spekker' Photoelectric Absorptiometer with Ilford (no. 607) orange filters. The continuous curve of Fig. 2 shows, for comparison, the extinction under the same conditions of pure, aqueous toluidine blue solutions of varying concentration. From these data the relation between the quantity of heparin added and the quantity of dye removed can be calculated,

and this is represented in Fig. 3. The proportion of dye removed increases in direct ratio to the concentration of heparin, until about 90% is combined; thereafter it increases more slowly, and a large excess of heparin is necessary for complete decolorization. Since there is no sharp end-point, a simple titration of heparin against dye is impracticable.

The relationship between the concentration of dye and the absorption of light of the chosen wave-length by aqueous toluidine blue solutions is not expressed by the Beer-Lambert law. The hue of this (and of other metachromatic dyes) varies with the concentration: in the case of toluidine blue, the absorption maximum shifts from the orange toward the yellow as the strength of the solution is increased. For this reason, it is impossible to match two watery solutions of the dye in a simple colorimeter of the Duboscq type if they differ in concentration by more than a few per cent. In the presence, however, of a suitable proportion of alcohol or acetone, the extinction curve of the dye corresponds to that of a very dilute aqueous solution, and is unaffected by changes in concentration. If, therefore, an equal volume of alcohol or acetone is added to the separated aqueous phase after extraction of the heparin-dye complex, the tint of the remaining dye is practically independent of its concentration, which may thus be measured directly with the aid of an ordinary colorimeter.

Factors modifying the reaction

Heparin and toluidine blue do not react in stoichiometric proportions. The quantity of dye changed to the metachromatic form depends on several factors besides the quantity of heparin present: it is diminished by an increase in temperature, in acidity, or in the concentration of other solutes in the system. Divalent and trivalent cations are especially active in reversing the reaction. In practice, however, these factors are easily controlled: the dilution of the material to be tested is so great that, except with very crude preparations, the influence of any impurities it may contain is negligible; and the disturbing effect of temperature changes can be avoided by carrying through the procedure with a standard and the unknown heparin simultaneously, so that the determination is comparative.

In particular, it may be noted that barium ions and tricresol (the preservative commonly added to heparin solutions) do not interfere, unless present in concentration higher than that of the heparin itself. The error introduced by a difference of temperature, at the moment of separating the heparin-dye complex from the unaltered dye, is about 1% for 1° C.

Colorimetric comparison of heparin preparations

Reagents.

(1) *0·005% toluidine blue solution.* 25 mg. of toluidine blue (Gurr's is satisfactory) is dissolved with the aid of heat in 500 ml. of 0·01 *N* HCl containing 0·2% of NaCl. The presence of acid minimizes the adsorption of the dye at glass surfaces.

(2) *Heparin* is dissolved in 0·2% NaCl. For purified samples a convenient concentration for both standard and unknown heparin is 0·01%. As some heparin preparations are nearly insoluble in distilled water, all aqueous solutions contain 0·2% of NaCl: the use of higher concentrations of NaCl tends to inhibit the reaction between heparin and the dye, and somewhat diminishes the accuracy of the estimation.

(3) *0·2% NaCl.*

(4) *Light petroleum*, B.P. 40–60°.

Procedure. 5 ml. of the toluidine blue solution are measured into each of nine 50 ml. test tubes. Varying amounts of a solution of the standard heparin (e.g. 0·2, 0·4, 0·8, 1·2 ml.) are added to tubes 1 to 4; corresponding amounts of the solution to be tested are added to tubes 5 to 8. The total volume is brought to 10 ml. in each tube by the addition of 0·2% NaCl, and the contents are mixed. 10 ml. of light petroleum are added to each tube, which is then stoppered and shaken vigorously for 30 sec. When separation of the two layers is complete, the aqueous phase is pipetted off from each tube, with care to avoid contamination by the heparin-dye complex precipitated at the interface. The absorption of each aqueous solution may be read immediately in the Spekker absorptiometer with orange (Ilford no. 607) filters. Alternatively, an aliquot may be diluted with an equal volume of alcohol and the reading made in an ordinary colorimeter, the toluidine blue solution containing no heparin (tube 9), and diluted with alcohol serving as a standard. Concentrations of the standard heparin are then plotted against absorptiometer (or colorimeter) readings, and a graph is drawn from which the relative affinity of the unknown heparin for the dye can be obtained. This may be conveniently expressed in terms of *colour units*, the *colour value* of the present provisional British standard heparin being taken as 110 units/mg. to correspond to its anticoagulant activity, which is defined as 110 units/mg. Thus, if 2 ml. of a solution of the unknown heparin removed the same amount of dye from solution as 1 ml. of a solution of equal concentration of the standard heparin, the 'colour value' of the unknown would be 55 units/mg.

From Fig. 3 it is evident that the colour value of a purified heparin can be measured with greatest precision when heparin is added in an amount sufficient to remove about 60–80% of the dye. With crude specimens, however, the impurities present may inhibit to a significant degree the formation of the heparin-dye complex, and the error so introduced will increase with increasing concentration of the impure heparin. It is therefore desirable to make the comparison between standard and unknown at different levels of heparin concentration, as described above. In this way, the graph will give, for each volume t of the test solution, a corresponding volume s of the standard solution, which produces the same colour change. If the ratio s/t is the same in each case, no difficulty arises; if (as with certain impure test samples), s/t decreases as s and t increase, it can be assumed that the highest value of s/t is the most nearly correct. It may be noted that, for all heparin samples so far examined which had been prepared by a process involving crystallization as the barium salt, s/t was found constant, within a few per cent, for all values of t.

Comparison of colorimetric and biological methods

The results obtained with a number of different materials are given in Table 1. The colorimetric comparison was carried out as described above, the biological comparison by the method described in the foregoing paper [MacIntosh, 1941]. The standard material was, in each case, the provisional British heparin standard, which has been defined as containing 110 anticoagulant units/mg. It has been taken to contain, by definition, 110 'colour units' per mg.

For all the samples of purified heparin tested, with one exception, the results of the two methods agreed within the limits of experimental error. The exception (no. 9 in Table 1) was a barium salt preparation, the activity of which had been deliberately reduced by prolonged treatment with acid alcohol: this showed reduced activity by both tests, but was about twice as active by the colorimetric as by the biological test. Sample no. 5 has been referred to in the preceding paper as giving significantly different results when compared with the standard

Table 1. *Anticoagulant activities and colour values of various materials*

(The provisional British heparin standard is taken to contain 110 anticoagulant units and 110 colour units/mg.).

No.	Description	Anticoagulant activity Units/mg.	Colour value Units/mg.
	Heparin		
1	Na salt (commercial)	106	110
2	Na salt (prepared from no. 5)	92	93
3	Na salt (commercial)	80	76
4	Na salt (commercial)	73	79
5	Ba salt (research specimen)	73	73
6	Ba salt (recrystallized from no. 5)	58	65
7	Ba salt (old sample)	30	35
8	Ba salt (from commercial Na salt)	68	72
9	Ba salt (acid-alcohol inactivated)	25	46
10	Crude dog heparin	20	23
11	Crude dog heparin	12	16
12	Crude dog heparin	12	8
	Other materials		
	'Liquoid' Roche (polyanethol-sulphonic acid)	50–60	46
	Chlorazol fast-pink	50–110	110
	Chlorazol sky-blue	30–75	75
	Bayer 205 (Germanin)	60	100
	Neosalvarsan	8–12	12
	Chondroitin sulphuric acid	<1	1
	Mucoitin sulphuric acid	<1	1

by two biological tests, the plasma-kinase and the rabbit's whole-blood methods: it is interesting to note that the colorimetric and plasma-kinase methods gave concordant results for this preparation.

The other anticoagulants tested were mostly synthetic preparations chemically very unlike heparin, and it was, consequently, not surprising to find that, in their effects on clotting, they differ qualitatively from heparin and from one another. Such differences were particularly noticeable with the dyes chlorazol fast-pink and chlorazol sky-blue [Huggett & Rowe, 1933], for which the slope of the curve relating concentration to clotting time was less steep than that found for heparin. The behaviour of Liquoid, Bayer 205 and neoarsphenamine in clotting experiments was not closely examined, but their anticoagulant action appears, likewise, not to be identical with that of heparin. Similar observations have been made by Astrup [1938]. The anticoagulant potencies given in Table 1 for these substances are therefore approximations only. As to the colorimetric comparison, it should also be noted that the activity of the synthetic anticoagulants, relative to that of heparin, was determined in only one set of conditions of temperature, salt concentration etc., and that different values might possibly have been found in other conditions.

Neoarsphenamine forms with toluidine blue a colourless complex which, like the red heparin-dye complex, is removed from aqueous solution by shaking with light petroleum.

Discussion

In the preceding paper it was pointed out that highly purified specimens of heparin, prepared by crystallization of the barium salt, are not necessarily identical in their specific anticoagulant activity. Not only are some preparations superior to others, to a degree not explained by differences in water or ash content, but the exact margin of this superiority varies, according to the test which is used to determine it. Heparin as ordinarily prepared is therefore not a

single substance, but contains two or more individuals whose activity appears to be unequally directed toward the different stages of the coagulation process.

These considerations make it obvious that the value of a colorimetric method for standardizing heparin can only rest on an empirical basis. It is obvious enough, from the results summarized in Table 1, that there is some sort of correlation between anticoagulant activity in general and the capacity for forming a complex with a metachromatic dye. What is not so clear is, whether a value found by the colour test may be taken to measure, with any degree of accuracy, the degree of a particular sort of anticoagulant activity, such as platelet-stabilizing, antiprothrombin or antithrombin. Still less is it clear—and a similar doubt attaches to the significance of the several biological assays—whether the particular activity, or combination of activities, measured by the colour reaction is actually that of most importance in the therapeutic use of heparin. While these points remain undecided, it is difficult or impossible to say that one assay method is better than another. So far as the available data go, one of the biological methods, the plasma-kinase test, appears to give results which agree remarkably well with those found by the colorimetric method, and better with these than with the results obtained by another biological method, the rabbit's whole-blood test. It may be that the first two measure chiefly one sort of anticoagulant activity and the latter another sort. It would not be unreasonable to suppose, for example, that an important factor in the delay by heparin of the clotting of the whole shed blood is due to its stabilization of the platelets, and the consequent delay in the liberation from these of thrombokinase. In the plasma test, on the other hand, this factor is eliminated, few if any platelets being present and kinase being, in any case, added artificially in excess; so that the delay by heparin of clotting in this system must be due to inhibition of the formation, or the action, of thrombin. Further speculation, however, along these lines is hardly profitable.

From the practical standpoint, the position is, perhaps, not so unsatisfactory as might appear from this discussion. It may probably be fairly described as follows (see also the preceding paper). If the standard preparation of heparin is of good quality (i.e. has suffered no appreciable denaturation during its isolation), then any good quality heparin may be compared with it, by any method, with the same result; if, however, the unknown preparation is of inferior quality, then its unit value will probably be higher by the whole-blood method than by the plasma-kinase or colorimetric method. Such experience as is now available suggests, however, that the various methods will rarely give results for samples of purified ox heparin differing by more than 25%. Differences of this magnitude are of little importance, except from the standpoint of cost; and, from any other point of view, there is a certain advantage in choosing the method giving the lowest value to a sample which has lost activity. Specimens prepared from other than ox tissues may be either more or less active [Jaques & Waters, 1940; Jaques, 1940], and it is possible that larger discrepancies in the results obtained by different methods may occur when these heparins from other species are compared with ox heparin. Until more information is available, however, there is much to be said for standardizing heparin by the simplest and most easily reproducible method yet available, and this would doubtless be a colorimetric method of the type here described.

SUMMARY

1. A simple colorimetric method for the standardization of heparin preparations is described, and its limitations are discussed. It gives results in good agreement with those obtained by the biological (plasma-kinase) method described in the preceding paper.

2. A number of synthetic anticoagulants have been compared with heparin by these two methods. In general, the relative potencies of different anticoagulants cannot be stated exactly, because of qualitative differences in their action.

I am indebted to Sir Henry Dale for his interest and for many valuable suggestions. My thanks are due to Dr S. A. Komarov of McGill University for samples of mucoitin and chondroitin sulphuric acids, and to Dr F. Bergel of Roche Products Ltd. for a sample of 'Liquoid' Roche. Some of the heparin samples were kindly supplied by Prof. A. R. Todd of the University of Manchester, Dr T. F. Dixon of British Drug Houses Ltd. and Mr W. A. Broom of Boots Pure Drug Co. Ltd.

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84. THE ACTION OF *l*-ASCORBIC ACID ON THE *IN VITRO* RESPIRATION OF LIVER TISSUE FROM GUINEA-PIGS ON A RESTRICTED DIET. I

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(*Received 25 June 1941*)

IN order to explore the possible connexion between *l*-ascorbic acid and tissue respiration, Harrison [1933] studied manometrically the respiration of tissues—mainly liver tissue—from normal and scorbutic guinea-pigs in the presence and in the absence of small quantities of *l*-ascorbic acid. He found that the addition of the vitamin to liver tissues of scorbutic animals increased the rate of O_2 uptake.

Quastel & Wheatley [1934], in investigating the effect of *l*-ascorbic acid on the oxidation of fats in the liver, found that slices from guinea-pigs fed on a scorbutic diet for 15 days or more usually showed an increased respiration in the presence of *l*-ascorbic acid which was significantly higher than that obtained with liver tissue from normal animals. The scorbutic animals employed by these workers after having reached a maximum weight eventually lost about 10–15% of the maximum value before being utilized for the experiment. Quastel & Wheatley thus obtained results somewhat similar to those of Harrison.

Phillips *et al.* [1934] were unable to confirm Harrison's results but it must be added that their technique differed widely from that employed by Harrison. For instance they utilized minced tissue in a phosphate-Ringer solution in air with 0·12 mg. of *l*-ascorbic acid per 200 mg. of tissue, whilst Harrison employed liver slices in a phosphate buffer solution in O_2 with 0·25 mg. of *l*-ascorbic acid per 200 mg. of sliced tissue.

Stotz *et al.* [1937] observed only irregular increases in the respiration on addition of *l*-ascorbic acid to liver slices from scorbutic and normal animals and they found no significant difference in behaviour between the tissue from normal and vitamin C-deficient guinea-pigs. They too used a medium different from that employed by Harrison.

It occurred to the present writers that the increased tissue respiration observed by Harrison might have been due not to the specific deficiency of vitamin C but to another dietetic restriction. Soon after the appearance of his communication we attempted to test this hypothesis experimentally, but in spite of various efforts, we were unable to reproduce Harrison's results. Nevertheless, Harrison's figures were so convincing that it became apparent that the discrepancy between his and our results must have been due to an undetected difference in technique. This was particularly probable, because Harrison used a differential correction for the oxidation of *l*-ascorbic acid in the medium which made the increases in his Q_{O_2} values appear to be lower than they really were. His correction was effected by introducing into the control flask of the Barcroft differential manometer a solution of ascorbic acid similar to that present in the

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flask containing the tissue. The aerobic oxidation of the added *l*-ascorbic acid in the control therefore proceeded at a higher rate than in the other flask owing to the absence of the protective action of the liver tissue. After a number of intermittent attempts, stretching over several years, we were eventually able to repeat Harrison's results with perfect regularity, but, as anticipated, we found that on addition of *l*-ascorbic acid an increase in the surviving respiration of the liver tissue could also be obtained when the animals had previously received a quantitatively restricted diet with an ample supply of vitamin C. The phenomenon is therefore not specific for scurvy in guinea-pigs.

Technique

Healthy growing guinea-pigs weighing 250–350 g. were used. The control or normally fed animals were offered a mixture of one part of oats and two parts of bran by volume and cabbage *ad lib.* Another group of animals received a diet restricted quantitatively so as to bring about some inanition, the quantity of the basal diet of oats and bran being varied daily so that they gradually lost weight and reached a steady value of *c.* 80% of their original weight after 15–20 days. The liver tissue was examined for its respiratory capacity in the presence and in the absence of ascorbic acid after the animals had subsisted on the restricted diet for 21–28 days. During this period, with the exception of the last 4–6 days when a daily dose of 10 mg. of ascorbic acid was given, the guinea-pigs received 15 g. of cabbage daily. These dietetically restricted animals therefore consumed daily a quantity of vitamin C which, although not high enough to saturate them fully, was many times higher than the dose required to protect them from macroscopic and microscopic lesions of scurvy and any difference observed in the respiration of their liver tissue could therefore not have been ascribed to scurvy. The third group of guinea-pigs received *ad lib.* a scorbutic diet made up of

Bran	6 parts, by volume	
Barley meal ...	2	,,
Middlings	3	,,
Fishmeal	1	,,
Crushed oats ...	4	,,

60 ml. of autoclaved milk made up from a dried powder were offered in addition to these animals.

Respiration was measured in a Warburg-Barcroft apparatus at 37° [cf. Dixon, 1934]. After stunning and bleeding the animals, the livers were excised and weighed. Tissue from corresponding lobes of the liver was cut with a razor blade and only thin slices (*c.* 0·3 mm.) were used. The cut slices were suspended in a shallow layer of buffer in a Petri dish and uneven or ragged ones were rejected. The suitable slices were then trimmed and cut into uniform pieces roughly 0·5 cm. across and an equal number, taken at random, was placed in each flask. This precaution was necessary since slices from different portions of the liver possess significantly different rates of respiration. In this way approximate uniformity of the mass of tissue in each flask was also ensured. As in Harrison's experiments the slices were suspended in *M*/15 Na-K phosphate buffer *p*H 7·4, the total volume of medium being kept at 2·5 ml. Manometer readings were taken every 10 min. during the 1st hr. and again at the end of the 2nd hr. After each experiment the *p*H of the medium was checked by capillator. The tissue was then removed for determination of dry weight and the contents of those flasks to which ascorbic acid had been added were rinsed out, acidified

and titrated with standardized 2:6-dichlorophenolindophenol. The O_2 uptake for each flask was calculated as μl. at N.T.P. per mg. of dried tissue. In each experiment 0·25 mg. of ascorbic acid was added to 4 of the 8 flasks containing the tissue and the mean of each 4 readings is recorded in the results.

Table 1. *The respiration of guinea-pig liver slices in the presence and absence of ascorbic acid*

Sex of animal	No. of days on diet	Weight g.			Mean Q_{O_2}					
					1st hr.			2nd hr.		
		Initial	Max.	Final	No addition	Ascorbate added	% increase	No addition	Ascorbate added	% increase
Normal diet										
F.	—	—	—	295	3·79	3·54	−6·6	2·92	3·54	21·2
M.	—	—	—	340	2·80	3·19	13·9	2·46	2·87	16·7
F.	—	—	—	350	4·40	4·78	8·6	—	—	—
M.	—	—	—	305	3·99	4·04	1·3	3·54	3·67	3·7
F.	—	—	—	320	2·92	3·57	22·3	2·88	3·46	20·1
F.	—	—	—	340	3·90	3·98	2·1	3·33	3·37	1·2
F.	—	—	—	360	3·46	4·54	31·2	2·98	4·11	37·9
F.	—	—	—	390	4·93	5·47	11·0	4·15	4·84	16·6
M.	—	—	—	350	4·07	4·36	7·1	3·17	3·49	10·1
M.	—	—	—	350	4·38	4·47	2·1	—	—	—
F.	—	—	—	260	6·44	6·33	−1·7	5·53	5·40	−2·3
F.	—	—	—	265	3·99	4·09	2·5	3·32	3·60	8·4
Mean	—	—	—	**327**	**4·09**	**4·36**	**7·8**	**3·43**	**3·84**	**13·4**
Scorbutic diet										
F.	26	260	320	270	3·55	5·12	44·2	—	—	—
M.	25	270	327	255	3·68	3·96	7·6	—	—	—
F.	26	275	305	210	3·49	3·92	12·3	—	—	—
M.	21	290	350	345	3·37	4·54	34·7	2·86	4·07	42·3
M.	21	290	320	260	3·86	5·17	33·9	3·24	4·54	40·1
M.	21	310	365	365	4·33	6·26	44·6	3·92	5·52	40·8
F.	21	290	350	265	4·17	5·84	40·0	3·54	5·01	41·5
M.	22	275	345	285	2·52	3·89	54·4	2·29	3·44	50·2
M.	21	270	355	315	2·83	4·05	43·2	2·42	3·44	42·1
F.	21	265	315	270	4·58	5·27	15·1	3·55	4·44	25·1
M.	21	280	360	345	3·77	5·34	41·7	2·97	4·45	49·8
M.	21	285	380	370	3·24	4·53	39·8	2·73	3·83	40·2
M.	21	275	335	335	4·24	5·99	41·3	3·42	4·94	44·4
M.	26	285	370	275	2·58	3·80	47·1	2·23	3·64	63·2
M.	27	300	405	287	2·61	4·20	60·9	2·22	3·64	64·5
Mean	—	**281**	**347**	**297**	**3·52**	**4·79**	**37·4**	**2·95**	**4·25**	**45·3**
Restricted diet										
		Initial wt.	Final wt.	% loss in wt.						
F.	—	275	200	27	3·91	5·46	39·6	3·11	4·65	49·5
M.	—	275	212	23	3·28	4·06	23·8	2·68	3·64	35·8
F.	—	300	220	27	5·77	6·62	14·7	4·92	5·66	15·3
M.	—	300	225	25	3·21	9·15	185·1	2·88	8·67	201·0
M.	—	300	222	26	3·63	4·95	26·5	2·91	3·66	25·8
F.	—	300	225	25	4·18	5·05	20·8	3·50	4·08	16·6
F.	—	295	230	22	3·24	5·24	61·7	2·57	4·20	63·3
M.	—	300	227	24	2·90	4·58	57·9	2·54	4·01	57·8
F.	—	285	215	25	4·45	5·78	29·9	3·75	5·66	51·0
F.	—	295	230	20	4·73	6·70	41·6	3·76	5·38	43·1
F.	—	280	230	18	4·45	4·68	5·2	3·73	3·93	5·4
F.	—	290	225	22	4·39	5·12	16·8	3·67	4·58	24·8
Mean	—	**291**	**222**	**24**	**4·01**	**5·62**	**43·6**	**3·33**	**4·84**	**49·1**

Results

The results are given in Table 1, from which it will be seen that the increase in respiration on the addition of ascorbic acid to liver slices of guinea-pigs on a normal diet was markedly less than occurred with those animals on a restricted diet or on a scorbutic diet: the rise in Q_{O_2} in these two groups were of the same order. These results will be discussed in conjunction with those reported in the following paper [Snow & Zilva, 1941]. It may perhaps be appropriate to stress here one point, namely that the *total* respiration of the liver tissue from the animals on the restricted diet after the addition of ascorbic acid was on the whole higher than that of the liver tissue from the animals on a normal diet. The bearing of this observation on the general problem will be considered later.

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85. THE ACTION OF *l*-ASCORBIC ACID ON THE *IN VITRO* RESPIRATION OF LIVER TISSUE FROM GUINEA-PIGS ON A RESTRICTED DIET. II

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(*Received 25 June 1941*)

In the preceding paper [Kellie & Zilva, 1941] it was shown that the rate of respiration of liver slices from guinea-pigs maintained on a quantitatively restricted diet containing an ample supply of vitamin C was considerably increased by the addition of *l*-ascorbic acid. In this communication this phenomenon is studied in greater detail.

Technique

Determination of thickness of liver slices. Since after a long trial it was found by us that the reproducibility of results greatly depended on the thickness of the liver slices employed, special care was taken to prepare sections of suitable and uniform thickness. This was periodically measured as follows. The moist weight of all the slices used in the experiment was first obtained by weighing the lobes before and after sectioning. Their area was then rapidly determined by spreading out flat in a shallow layer of buffer solution in a Petri dish placed on a piece of photographic bromide paper and exposing to a light above the dish. A superimposed exposure was then made through a piece of translucent graph paper. After development the area of the slices was obtained by counting the number of squares in the image of each slice. The density of the liver was determined by weighing a portion in air and in water; as this value (1·09) was found to be very constant, it was unnecessary to repeat the determination in each experiment. The mean thickness was then calculated by dividing the total moist weight of the slices by the product of their area and the density.

The accuracy of this method was checked by the following two independent procedures.

(1) The mean thickness of a group of slices was obtained from measurements of the thickness of a lobe before and after sectioning by means of a micrometer gauge.

(2) Individual slices were measured by means of the focussing vernier on a microscope and the mean thickness then calculated.

Results from the three methods are compared in Table 1 and it will be seen that there is satisfactory agreement between them.

Table 1. *Slice thickness determination by various methods*

Mean thickness of slices in mm.		
Weighing	Screw gauge	Microscope
0·32	—	0·35
0·37	0·32	0·32
0·30	0·35	0·28

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Manometric technique. The manometric technique was essentially the same as that described [Kellie & Zilva, 1941] except that 1·5 ml. instead of 2·5 ml. of medium containing $10^{-3}M$ ascorbic acid were used in these experiments. As before, a freshly prepared $M/15$ KH_2PO_4 solution adjusted to *p*H 7·4 with NaOH was used as a buffer. It must, however, be pointed out here that the constitution of the buffer is not without influence on the effect which ascorbate exercises on the respiration of the liver slices, as will be seen from the results obtained when 'physiological phosphate saline' [Krebs & Henseleit, 1933] was used as medium (Table 2). Whilst the respiration of the liver tissue from normal animals was

Table 2. *Respiration of guinea-pig liver slices in Krebs' physiological saline*

Volume of medium 1·5 ml. Conc. of ascorbate $10^{-3}M$ unless otherwise stated

Animals receiving restricted diet						Animals receiving normal diet		
Mean Q_{O_2} values		% increase in Q_{O_2} due to ascorbate	Mean Q_{O_2} values		% increase in Q_{O_2} due to ascorbate	Mean Q_{O_2} values		% increase in Q_{O_2} due to ascorbate
No addition	Ascorbate added		No addition	Ascorbate added		No addition	Ascorbate added	
3·44	3·51	2	1·80	2·20	22	5·97†	5·81	−3
3·25*	3·57	10	2·68	2·58	−4	4·19†	4·24	1
3·78*	4·45	18	2·08	2·48	19	7·16†	7·24	1
2·69*	2·73	1	2·34	3·20	37	4·80	4·64	−3
2·45	2·88	18	2·56	2·82	10	4·77	4·76	0

* In these exps. volume of buffer was 2·5 ml.
† In these exps. conc. of ascorbic acid was $0·5 \times 10^{-3}M$.

not affected in this case by the addition of ascorbate, as is usual in the above phosphate buffer, the increase in Q_{O_2} (the minus sign is omitted in all references to Q_{O_2} in this paper) of the tissues from the animals on the restricted diet was however neither so great nor so consistent as that obtained when the neutralized $M/15$ KH_2PO_4 buffer solution was used. The different constitution of the buffer was in all probability responsible for the discrepancy in the results obtained by various workers [cf. Phillips *et al.* 1934 and Stotz *et al.* 1937].

The output of CO_2 was determined by the Warburg method as described by Dixon [1934], in an atmosphere of O_2 and in the same volume of phosphate buffer as that used for measurement of O_2 uptake. The CO_2 was liberated by 0·3 ml. *N* HCl contained in the side bulbs and the same amount of acid was also added at the end of the experiment to the flasks in which the O_2 uptake measurements were made, in order to obviate the effect of any possible change in weight of the dried tissue. Two control flasks containing buffer and buffer and tissue respectively were also included. All measurements were carried out over a period of 1 hr. since it was found by Kellie & Zilva [1941] that measurements over longer periods did not significantly alter the trend of the experiment.

Reagents. (Melting points uncorrected.)

Ascorbic acid—99–100% purity. Messrs Roche Products Ltd.

Reductic acid—was prepared from pectic acid by Reichstein & Oppenauer's method [1933]. Twice sublimed. Yellowish-white crystals. M.P. 209° (decomp.).

d-Glucoascorbic acid—sample kindly presented by Prof. E. L. Hirst, purity *c.* 85%. Brownish amorphous powder.

Malonic acid—commercial sample twice recryst. from ethyl acetate. White needles. M.P. 135·5°.

Hydroxymalonic acid—commercial sample recryst. from a mixture of ethyl acetate and light petroleum. White anhydrous crystals M.P. 156°. The purification is especially important because commercial samples appear to contain some impurity which acts as a substrate to liver slices.

Iodoacetic acid—commercial sample recryst. from light petroleum. White crystals M.P. 83°.
Maleic acid—commercial sample recryst. from ethyl acetate. White crystals M.P. 135°.
Arsenious oxide—pure commercial sample, Kahlbaum.
Phloridzin—commercial sample recryst. twice from water and dried *in vacuo* over H_2SO_4; faintly yellowish crystals M.P. 213° (decomp.).
Sodium fluoride—Puriss. commercial sample.
Sodium pyrophosphate—commercial sample twice recryst. from water.
Cinnamic acid—commercial sample. M.P. 133·5°.
Potassium cyanide—AnalaR.

Increase in respiration on addition of ascorbate to liver slices from guinea-pigs on restricted diet as compared with respiration of liver slices of the same thickness from animals on a normal diet

The texture of the livers from animals on a normal diet differs considerably from that of the livers of those maintained on a restricted diet. In order to demonstrate beyond all doubt that the differential effect of the addition of ascorbate to the liver tissue from guinea-pigs on the two diets observed by Kellie & Zilva [1941] was not due to an accidental difference between the thickness of the slices of the two groups of animals, the thickness of the slices used in this experiment was measured (Table 3). Although the thickness was of the

Table 3

Animals receiving normal diet		Animals receiving restricted diet	
Mean thickness of slices mm.	Mean % increase in Q_{O_2} due to added ascorbate	Mean thickness of slices mm.	Mean % increase in Q_{O_2} due to added ascorbate
0·33	−2	0·29	38
0·27	1	0·28	35
0·32	−4	0·31	22
0·29	−3		

same order in both cases, the liver tissue of the guinea-pigs on the restricted diet unlike that from the animals on the normal diet showed as before an increase in the Q_{O_2} on addition of ascorbate. Evidently the empirical method of cutting the liver tissue adopted by us yielded slices of similar thickness (approximately 0·3 mm.) both from guinea-pigs on a normal and on a restricted diet. This reproducibility was further confirmed by occasional measurements.

Influence of the age of animals used

As already mentioned the guinea-pigs normally used in all experiments were approximately 300 g. in weight. Animals of this weight are still growing rapidly and it was of interest to find out whether liver slices from fully grown guinea-pigs gave similar results to those from younger animals. Adult guinea-pigs about 8 months old which had been kept on a mixed diet were used. One group was kept on the normal diet previously described and another group placed on the restricted diet similar to that used for the younger animals. The loss in weight during the restriction of the diet (20–25 days) was of course relatively less. From results given in Table 4 it will be seen that liver slices from adult guinea-pigs on a restricted diet respire very slowly but the Q_{O_2} is nevertheless greatly increased by addition of ascorbate; the effect is even more marked than in the case of young guinea-pigs. It is further seen that although liver slices from adult animals on a normal diet respire more vigorously than those from animals on a restricted diet the Q_{O_2}, as in growing animals, is not increased by addition of ascorbate.

Table 4. *Respiration of liver slices from adult guinea-pigs*

Medium—1·5 ml. phosphate buffer. Ascorbate concentration 10^{-3} *M*

Animals receiving normal diet				Animals receiving restricted diet				
	Mean Q_{O_2} values		% increase in Q_{O_2} due to added ascorbate	Wt. of animal, g.		Mean Q_{O_2} values		% increase in Q_{O_2} due to added ascorbate
Wt. of animal g.	No addition	Ascorbate added		Before restriction	After restriction	No addition	Ascorbate added	
905	1·80	1·82	1	855	710	1·68	4·87	190
740	5·11	5·18	1	855	687	1·56	4·04	159
720	4·09	4·12	1	865	650	1·87	4·52	142

Influence of ascorbate concentration

Liver slices from guinea-pigs on a restricted diet were allowed to respire in the presence of various concentrations of ascorbate. As it was found to be impossible for us to investigate the effect of more than three or four concentrations in any one experiment it was difficult to obtain as clear a picture of the response of O_2 uptake to a wide range of concentrations of ascorbate as if the experiments were carried out in one series. However, certain deductions can be drawn from the results of a number of such experiments performed at different times (Table 5). First, it must be pointed out that the amount of ascorbate

Table 5. *Effect of varying concentrations of ascorbate on the respiration of liver slices from guinea-pigs on restricted diet*

Medium—1·5 ml. phosphate buffer

Concentration of ascorbate added m*M*	Mean Q_{O_2}	Increase in Q_{O_2} due to added ascorbate	Mean dry wt. of tissue mg.	Added ascorbic, μg. / Tissue dry wt., mg.	Concentration of ascorbate added m*M*	Mean Q_{O_2}	Increase in Q_{O_2} due to added ascorbate	Mean dry wt. of tissue mg.	Added ascorbic, μg. / Tissue dry wt., mg.
0	5·12	—	19·6	—	0	2·36	—	12·1	—
0·04	5·67	0·55	18·6	0·6	0·04	3·02	0·66	10·3	1·0
0·10	5·66	0·54	17·4	1·5	0·10	3·33	0·97	12·3	2·1
0·20	6·38	1·26	17·1	3·1	0·20	4·16	1·80	11·6	4·6
0	2·95	—	15·2	—	1·0	6·03	3·67	9·1	29
0·05	3·91	0·96	16·0	0·8	0	3·30	—	11·2	—
0·10	4·19	1·24	16·3	1·6	0·01	2·90	(−0·40)	12·6	—
0·15	4·50	1·55	14·2	2·8	0·10	4·07	0·77	12·4	2·1
0	2·46	—	19·9	—	1·0	5·59	2·29	11·0	24
0·04	2·66	0·20	18·6	0·6	0	2·76	—	10·5	—
0·10	2·91	0·45	17·2	1·5	0·2	4·16	1·40	11·8	4·5
0·20	3·38	0·92	20·2	2·6	0·6	4·91	2·15	12·7	12
1·0	4·37	1·91	17·1	15	2·0	5·41	2·65	9·9	53
0	3·04	—	9·6	—	0	3·50	—	16·4	—
0·04	4·02	0·98	9·8	1·1	1·0	6·47	2·97	13·1	20
0·10	4·81	1·77	8·1	3·3	1·5	7·10	3·60	9·9	40
0·20	5·07	2·03	10·4	5·1	2·0	6·82	3·32	11·9	44

present per unit mass of tissue is of greater significance than the actual concentration of ascorbate in the flasks. In Table 5 a column is therefore given showing the μg. ascorbic acid per mg. dried tissue, which will be referred to as the 'relative concentration of ascorbate'.

It will be observed that even quite low relative concentrations of ascorbate produced definite increases in the O_2 uptake of the tissue. At low relative concentrations increasing amounts of ascorbate produced a more pronounced increase than at higher levels and when a relative concentration of more than 20 was

reached the Q_{O_2} almost ceased to be influenced by an increase in the amount of ascorbate present. These higher concentrations thus represent a limit to the magnitude of the increase in the O_2 uptake which may be produced by the addition of ascorbate. The curve connecting the increase in Q_{O_2} with relative vitamin concentrations is probably similar to those obtained when the effect of the concentration of a coenzyme on its rate of hydrogenation is studied [cf. Negelein & Haas, 1935; Negelein & Wulff, 1937]. In all our subsequent experiments a relative concentration of the order of 18 was employed. Under these conditions an almost maximal increase in the Q_{O_2} is obtained and small fluctuations such as occur owing to the unavoidable variation in the weight of tissue in the various flasks in any particular experiment have little effect on the results.

Influence of compounds chemically related to ascorbic acid on the O_2 uptake by liver slices from guinea-pigs on a restricted diet

Two such compounds, *d*-glucoascorbic acid, an antiscorbutically inactive homologue of *l*-ascorbic acid and reductic acid, a compound also containing a dienol group, were investigated. Both these compounds possess a reduction potential similar to that of *l*-ascorbic acid. Three sets of flasks were used in each experiment: these contained (1) no addition, (2) *l*-ascorbate and (3) an equimolar quantity of reductate or *d*-glucoascorbate. Thus there was always a direct comparison between *l*-ascorbate and the other compounds. The results in Table 6

Table 6. *Influence of ascorbic acid analogues on the respiration of liver slices from guinea-pigs on a restricted diet*

Medium—1·5 ml. phosphate buffer

Concentration of added compounds (M)	Mean Q_{O_2} values			% increase in Q_{O_2} due to		Loss of added substances in μ-mol./mg. dried tissue	
	No addition	*l*-Ascorbate	Reductate	*l*-Ascorbate	Reductate	*l*-Ascorbate	Reductate
10^{-3}	2·78	4·18	3·91	50	41	0·028	0·043
10^{-3}	3·48	4·31	4·14	24	19	0·017	0·008
10^{-4}	2·59	4·16	3·95	61	53	—	—
10^{-4}	2·81	4·04	3·53	44	26	—	—
	No addition	*l*-Ascorbate	*d*-Glucoascorbate	*l*-Ascorbate	*d*-Glucoascorbate	*l*-Ascorbate	*d*-Glucoascorbate
10^{-3}	2·52	3·07	3·32	22	32	0·018	0·026
10^{-3}	2·08	2·48	2·40	19	15	0·021	0·015
10^{-4}	3·39	4·15	4·52	22	33	—	—
10^{-4}	3·88	4·81	4·76	24	23	—	—

show that both reductate and *d*-glucoascorbate bring about an increase in the Q_{O_2} of liver slices from guinea-pigs on a restricted diet. Moreover, the increases observed were of a magnitude similar to that obtained with *l*-ascorbate even when the concentration of the added substances was as low as $10^{-4}M$. The increase in the respiration is therefore not specific for *l*-ascorbic acid and is independent of the antiscorbutic activities of the compounds; it seems rather to be controlled by their reduction potentials. In this and in the following experiments the loss of the added ascorbic acid during the respiration period is given. The bearing of these figures on the general aspect of the problem will receive reference in the discussion.

The influence of 'saturation' with simultaneous food restrictions on the respiration phenomena associated with added ascorbic acid

Although the experimental guinea-pigs on the restricted diet used in this investigation received daily quantities of vitamin C several times higher than the dose necessary to protect them from macroscopic and microscopic lesions of scurvy, they were not fully saturated in respect of vitamin C. The control animals on the normal diet were of course fully saturated. It was desirable to ascertain whether the increase in the respiration of the liver tissue from restricted animals on addition of ascorbic acid might possibly have been due to the partial saturation of the animals, and with this purpose in view the following experiment was performed. One group of animals on a restricted diet received daily 30–50 mg. of ascorbic acid *per os* over a period of 10–14 days, whilst in another group 50 mg. of neutralized ascorbic acid were injected into the femoral muscle 24 hr. before the respiration of the liver tissue was studied. These conditions are known to produce full saturation [cf. Zilva, 1936]. The results in Table 7 show

Table 7. *Respiration of liver slices from guinea-pigs on a restricted diet receiving large doses of ascorbate*

Medium—1·5 ml. phosphate buffer. Ascorbate concentration 10^{-3} *M*

Dose ascorbic acid (neutralized) mg./day	Method of administration	Period of dosage days	Mean Q_{O_2} values		% increase in Q_{O_2} due to added ascorbate	Loss of ascorbic μg./mg. dried tissue
			No addition	Ascorbate		
30	Oral	10	3·46	5·39	56	6·1
30	,,	11	3·58	5·36	50	7·1
50	,,	12	6·30	7·56	20	4·7
50	,,	13	5·00	5·42	8	4·6
50	,,	14	6·23	5·85	−6	3·6
50	,,	12	3·72	4·80	29	5·4
50	Injection	—	4·54	5·00	10	3·2
50	,,	—	4·72	6·39	35	5·9
50	,,	—	3·90	4·85	24	3·6
50	,,	—	2·04	2·50	23	3·8
50	,,	—	2·49	4·25	71	7·0
50	,,	—	3·23	3·82	18	4·0

that there was such an increase in the Q_{O_2} in the majority of cases when ascorbic acid was added to the tissues that no doubt is left that the enhanced respiration which took place under these circumstances was due to a dietetic restriction as such and not to a state of partial vitamin C saturation.

The relationship between O_2 uptake and CO_2 output

It has been shown on the one hand that the livers of about 30% of animals on a normal diet showed a significant increase in Q_{O_2} in the presence of added ascorbate [Kellie & Zilva, 1941] and that on the other hand under the same conditions only 8% of the livers of animals on the restricted diet failed to show an increase in Q_{O_2}. Therefore the results which deviated from the majority in each group were eliminated from these as well as from the experiments in the next section in order to avoid complications.

Although the Q_{CO_2} figures (Table 8) were extremely variable in the case of the animals receiving the restricted diet, the increase in the volume of CO_2 evolved when it did occur was much lower (2/5–1/10) than the O_2 taken up. It would therefore appear that the respiration stimulated by the addition of the ascorbate

Table 8. *Effect of added ascorbate on the Q_{O_2} and Q_{CO_2} of respiring guinea-pig liver slices*

Medium—1·5 ml. phosphate buffer. Ascorbate concentration 10^{-3} *M*

Mean Q_{O_2} values		% increase in Q_{O_2} due to added ascorbate	Mean Q_{O_2} values		% increase in Q_{CO_2} due to added ascorbate
No addition	Ascorbate		No addition	Ascorbate	
		Restricted diet			
3·70	5·66	53	3·16	3·36	6
2·97	6·11	106	2·27	1·64	−28
2·70	4·86	80	1·32	1·86	41
3·98	5·85	47	2·48	3·17	28
5·22	6·09	17	3·71	3·84	4
3·74	5·23	40	2·31	1·88	−19
		Normal diet			
6·40	6·17	−4	4·97	4·51	− 9
3·17	3·39	7	2·09	2·24	7

did not involve complete combustion. As would be expected no alteration was observed in the CO_2 output or in the O_2 uptake in the two control animals in this experiment.

The influence of inhibitors on the respiration of liver slices from guinea-pigs on a normal diet and from animals on restricted diet

In order to throw some light on the mechanism involved in the production of the increased oxidation by the addition of ascorbic acid to liver slices from animals kept on a restricted diet, the influence of a range of inhibitors on the respiration of liver slices from animals on a restricted diet as well as from animals subsisting on a mixed diet was investigated. The inhibitors chosen were substances known to modify the catabolism of carbohydrates and some of them have been shown to arrest specific reactions in the Embden-Meyerhof and other schemes of intermediary metabolism. The lowest concentrations previously found by other workers in this field to produce marked inhibition were employed. The results are given in Table 9.

Phloridzin. Phloridzin greatly reduced the Q_{O_2} of slices from animals on a restricted diet, and also largely abolished the increase in the Q_{O_2} brought about by ascorbate. The reduction in the Q_{O_2} also occurred in slices from normal animals, but in this case the addition of ascorbate had no appreciable influence either in the presence or absence of phloridzin.

Iodoacetate. The action of iodoacetate on guinea-pig liver slices caused a marked reduction in the Q_{O_2} of slices from animals on a normal diet. In the presence of ascorbate however iodoacetate was almost without influence [cf. Quastel & Wheatley, 1934]. With animals on a restricted diet quite different results were obtained. In this case in the absence of ascorbate, iodoacetate caused very little decrease in the Q_{O_2} of the slices. In the presence of ascorbate however, the addition of iodoacetate caused a rise in the Q_{O_2} higher than that obtained by the addition of ascorbate alone as will be seen from the negative values shown in Table 9 for the % inhibition. It will be seen (Fig. 1) that the typical curves for the O_2 uptake in the presence of ascorbate or ascorbate and iodoacetate in the case of the liver of guinea-pigs on a normal diet are almost coincident with those for the untreated tissue. The O_2 consumption of tissue poisoned with iodoacetate falls off much more noticeably than that of the unpoisoned slices. In the case of the animals on the restricted diet it will be

Table 9. *Action of inhibitors on the respiration of liver slices from guinea-pigs on normal and restricted diets*

O = no addition. A = ascorbate (10^{-3} *M*) added. I = inhibitor.

Medium—1·5 ml. phosphate buffer

Inhibitor	Concentration of inhibitor	Nature of diet	Mean Q_{O_2} values				% increase due to A		% inhibition due to I		Loss of ascorbate in μg./mg. dried tissue	
			O	A	I	A+I	No I	I	No A	A	No I	I
Phloridzin	10^{-2} *M*	Normal	5·31	5·19	2·48	2·45	−2	−1	53	53	2·3	3·2
,,	,,	,,	3·91	3·84	1·82	2·02	−2	11	53	47	2·8	2·9
Phloridzin	10^{-2} *M*	Restricted	3·66	7·18	1·59	1·99	96	25	57	72	15·9	7·3
,,	,,	,,	3·09	6·09	1·82	2·19	97	20	41	64	10·6	5·8
,,	,,	,,	2·77	3·40	1·33	1·32	23	−1	52	61	4·7	4·4
Iodoacetate	10^{-3} *M*	Normal	2·54	2·44	1·41	1·97	−4	40	44	19	2·7	4·3
,,	,,	,,	6·46	6·19	4·14	6·55	−4	58	36	−6	4·0	9·9
,,	,,	,,	3·56	3·53	2·85	3·55	−1	25	20	−1	3·5	7·2
,,	,,	,,	5·28	5·16	3·92	5·44	−2	39	26	−5	2·6	7·0
Iodoacetate	10^{-3} *M*	Restricted	2·87	5·80	2·82	5·81	102	106	2	0	—	—
,,	,,	,,	2·73	5·36	2·53	6·06	96	140	7	−13	11·1	12·1
,,	,,	,,	3·47	5·38	3·49	6·62	55	90	−1	−23	8·3	11·6
,,	,,	,,	2·65	3·60	2·40	5·98	36	149	9	−66	9·8	16·5
,,	,,	,,	4·21	6·89	3·48	8·17	64	135	17	−19	10·9	19·0
,,	,,	,,	3·54	4·32	2·75	5·42	22	97	22	−25	4·7	9·3
Fluoride	2×10^{-2} *M*	Normal	2·60	2·65	2·25	2·48	2	10	13	6	3·7	3·8
,,	,,	,,	5·00	5·28	2·93	3·73	6	27	41	29	3·5	4·0
,,	,,	,,	3·44	3·32	2·47	2·63	−3	6	28	21	2·4	3·3
Fluoride	2×10^{-2} *M*	Restricted	1·92	3·80	1·27	3·66	98	188	34	4	5·5	6·4
,,	,,	,,	2·70	6·11	1·84	5·43	126	195	32	11	13·2	12·1
,,	,,	,,	2·67	2·80	2·17	2·64	5	22	19	6	3·7	4·2
,,	,,	,,	3·14	5·31	2·68	5·29	69	97	15	0	8·2	8·7
,,	,,	,,	2·21	5·72	1·64	5·42	159	230	26	5	12·7	12·8
Maleate	2×10^{-2} *M*	Normal	3·34	3·42	2·73	3·04	2	11	18	11	3·2	2·7
,,	,,	,,	4·70	4·20	4·39	3·93	−11	−10	7	6	3·4	3·1
,,	,,	,,	4·16	4·22	3·00	3·11	1	4	28	26	2·1	2·8
Maleate	2×10^{-2} *M*	Restricted	2·11	4·41	1·74	4·29	109	147	18	3	8·6	9·2
,,	,,	,,	3·34	6·14	2·75	5·50	84	100	18	10	9·5	10·2
,,	,,	,,	2·59	5·08	2·11	4·72	96	124	19	7	7·8	8·0
Arsenite	10^{-3} *M*	Normal	2·99	3·07	1·61	2·20	3	37	46	28	2·6	4·6
,,	,,	,,	4·99	4·91	3·24	4·21	−2	30	35	14	—	—
,,	,,	,,	3·43	3·27	1·52	1·68	−5	11	56	49	2·2	2·7
Arsenite	10^{-3} *M*	Restricted	2·30	4·04	1·94	3·74	76	93	16	7	6·8	6·9
,,	,,	,,	3·03	4·81	2·02	4·10	59	103	33	15	9·8	12·6
,,	,,	,,	4·01	4·36	1·71	2·82	9	65	57	35	4·3	6·2
Malonate	10^{-2} *M*	Normal	3·36	3·42	2·65	3·14	2	18	21	8	3·8	4·8
,,	,,	,,	4·32	4·16	3·90	3·57	−4	−8	10	14	2·2	2·1
,,	,,	,,	2·77	2·69	2·31	2·32	−3	0	17	14	2·8	3·1
Malonate	10^{-2} *M*	Restricted	3·43	6·71	3·46	6·27	96	81	−1	7	7·3	8·1
,,	,,	,,	2·41	5·39	2·19	4·73	124	116	9	12	9·8	8·7
,,	,,	,,	3·48	6·62	2·89	5·98	90	107	17	10	11·3	12·5
Pyrophosphate	2×10^{-2} *M*	Normal	6·32	6·00	4·61	4·91	−5	7	27	21	3·0	1·1
,,	,,	,,	4·99	4·85	4·20	3·89	−3	−7	16	20	2·8	1·1
Pyrophosphate	2×10^{-2} *M*	Restricted	2·58	4·50	1·83	1·93	74	5	29	57	6·0	2·0
,,	,,	,,	3·20	6·87	2·23	2·63	115	18	30	62	12·4	4·0
,,	,,	,,	2·56	4·89	1·59	1·97	91	24	38	60	9·1	3·7
Hydroxymalonate	2×10^{-2} *M*	Normal	5·32	5·49	4·28	4·53	3	6	20	17	2·4	2·8
,,	,,	,,	6·98	6·99	4·92	5·25	0	7	30	25	—	—
Hydroxymalonate	2×10^{-2} *M*	Restricted	2·86	4·68	2·68	2·16	64	−19	6	54	6·9	3·2
,,	,,	,,	3·18	4·38	2·63	2·48	38	−6	17	43	5·6	4·2
,,	,,	,,	2·96	4·02	2·42	2·50	35	3	18	38	5·0	3·3
Cyanide	10^{-3} *M*	Normal	3·25	3·56	2·33	2·30	10	−1	28	35	2·9	2·9
,,	,,	,,	4·25	4·17	2·49	2·66	−2	7	41	36	2·8	3·3
Cyanide	10^{-3} *M*	Restricted	2·64	4·30	1·00	1·75	63	75	62	59	7·6	5·0
,,	,,	,,	2·72	3·24	1·38	1·62	19	17	49	50	3·8	3·3
Cinnamate	10^{-3} *M*	Restricted	2·60	3·65	2·50	3·34	40	34	4	8	6·6	6·2
,,	,,	,,	2·69	3·44	2·62	3·47	28	32	3	−1	7·3	4·9

noticed that although after 60 min., the tissue poisoned with iodoacetate has absorbed less O_2 than the untreated material, the presence of iodoacetate produced a slight initial increase in O_2 consumption compared with the control. This effect was observed in five out of six of the experiments performed with animals on a restricted diet but with animals on a normal diet it was not once observed.

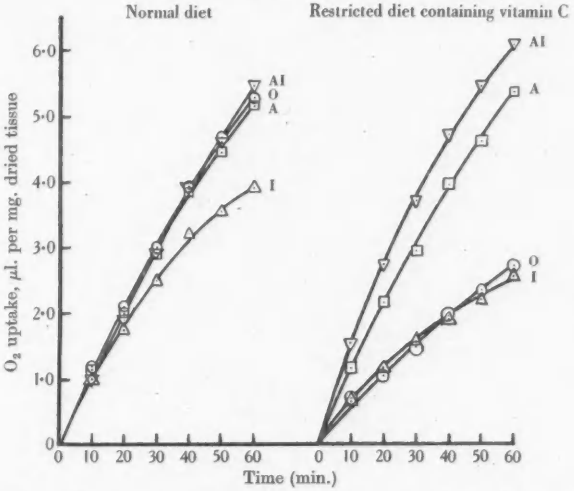

Fig. 1. Influence of iodoacetate and ascorbate on the course of respiration of liver slices from guinea-pigs maintained on normal and restricted diets. O, ⊙—⊙ Tissue only. I, △—△ 10^{-3} *M* iodoacetate. A, ⊡—⊡ 10^{-3} *M* ascorbate. AI, ▽—▽ 10^{-3} *M* ascorbate + 10^{-3} *M* iodoacetate.

It may be added that ascorbate kept without tissue under the same experimental conditions in the presence of iodoacetate with or without glutathione remained unchanged.

Fluoride. Fluoride brought about some reduction in the Q_{O_2} of liver slices from animals on a normal diet in the presence and in the absence of ascorbate. With animals on a restricted diet the presence of ascorbate prevented the inhibition by fluoride to a large extent.

Maleate. Maleate caused some reduction in the Q_{O_2} of slices from animals on both normal and restricted diets. Ascorbate had no significant effect on the former, but in the case of the latter the extent of the inhibition was possibly somewhat reduced.

Arsenite. Arsenite brought about a marked reduction in the Q_{O_2} of slices from guinea-pigs on both normal and restricted diets. When ascorbate was also added, the inhibiting effect was somewhat diminished in the case of both groups of animals.

Malonate. The results of the effect of malonate on the liver slices from animals on normal and restricted diets show that the reduction in the Q_{O_2} was not great in either case, and the presence of ascorbate made no difference to this minor inhibiting effect.

Pyrophosphate. Pyrophosphate reduced the Q_{O_2} of liver slices from animals on a normal diet and the addition of ascorbate had no effect either in the

presence or in the absence of the inhibitor. When animals on the restricted diet were employed, an inhibitory effect was also observed but the pyrophosphate almost entirely abolished the increase in Q_{O_2} brought about by the action of ascorbate. In this respect its behaviour was similar to that of phloridzin.

Hydroxymalonate. This inhibitor reduced the respiration of liver slices of animals on both normal and restricted diets. In the latter, however, hydroxymalonate entirely eliminated the usual increase brought about by the addition of the ascorbate.

Cyanide. In experiments with cyanide, a cyanide solution was placed in the central cup for the absorption of CO_2 in order to avoid loss of HCN from the buffer medium [Krebs, 1935]. Cyanide produced large inhibitions in the Q_{O_2} of slices from animals on normal and restricted diets but the addition of ascorbate caused nevertheless the same relative increase in Q_{O_2} of the slices from animals on a restricted diet whether or not the tissue was poisoned with cyanide. It therefore did not influence the particular respiratory process which was accelerated by ascorbate.

Cinnamate. Cinnamate had no appreciable effect upon the Q_{O_2} values either in the presence or in the absence of ascorbate when liver tissue from guinea-pigs on a restricted diet was used.

Discussion

The experiments described in this and the preceding communication show beyond doubt that the addition of ascorbic acid to liver slices from guinea-pigs maintained for some time on a quantitatively restricted diet increases the rate of the surviving respiration of this tissue. This occurs when the animals receive at the same time sufficient vitamin C to protect them from scurvy or even to saturate them with the vitamin and consequently the phenomenon seems to have no connexion with the development of scurvy. Furthermore, the stimulating action on the respiration is not confined to ascorbic acid alone but can be brought about by the presence of chemically related compounds such as *d*-glucoascorbic acid and reductic acid which possess a similar reduction potential but no antiscorbutic activity.

Provided that the necessary experimental conditions, particularly as regards the buffer and thickness of slices, are observed the results are definitely reproducible. We have a record of 106 experiments with guinea-pigs on a restricted diet and 67 experiments with animals on a normal diet. The surviving respiration of the liver tissues of 95 % of the former and of only 31 % of the latter responded to the addition of ascorbic acid by an increase in the rate of respiration. That this was not controlled by the ascorbic acid content of the livers was shown by the fact that the quantity of the vitamin in the flasks when livers of normal and consequently saturated animals were used was well below, about a tenth, the quantity of added ascorbic acid required to produce the maximum increase in O_2 uptake, and the livers of normal animals should therefore have reacted to the addition of ascorbic acid. In spite of this, only 31 % of the livers from the saturated animals on a normal diet responded to the addition of ascorbic acid. Additional point is lent to this view by the fact that, in the case of the livers of guinea-pigs saturated with vitamin C, 10 out of 12, i.e. 83 %, were found to respond to this addition. Furthermore, as was noted by Kellie & Zilva [1941], the livers from restricted animals respired on an average more vigorously after the addition of ascorbic acid than did those from normal animals.

The question arises whether the increase in the O_2 uptake was due to the oxidation of some of the added ascorbic acid or to an increase in the rate of

tissue respiration brought about by this addition. A correlation of a number of our results, selected at random from the preceding tables and arranged in the ascending order of the increase in Q_{O_2} (Table 10), does not favour the former

Table 10. *The relationship between the loss of added ascorbate and the increase in Q_{O_2} of respiring liver slices from guinea-pigs on restricted diet*

Increase in Q_{O_2} due to added ascorbate	Gross relative loss*	Corrected relative loss*	Molar ratio†	Increase in Q_{O_2} due to added ascorbate	Gross relative loss*	Corrected relative loss*	Molar ratio†
0·13	3·7	0·7	1·5	2·30	8·6	5·6	3·2
0·42	3·6	0·6	5·5	2·33	9·1	6·1	3·0
0·78	4·7	1·7	3·6	2·49	7·8	4·8	4·1
0·95	9·8	6·8	1·1	2·68	10·9	7·9	2·7
1·05	6·6	3·6	2·3	2·80	9·5	6·5	3·4
1·06	5·0	2·0	4·2	2·98	9·8	6·8	3·4
1·20	5·6	2·6	3·6	3·00	10·6	7·6	3·1
1·74	6·8	3·8	3·6	3·28	7·3	4·3	6·0
1·82	6·9	3·9	3·7	3·41	13·2	10·2	2·6
1·88	5·5	2·5	5·9	3·67	12·4	9·4	3·1

* 'Relative loss of ascorbate' $= \dfrac{\text{Loss of ascorbic acid } (\mu\text{g.})}{\text{Dry wt. of tissue (mg.)}}$.

† 'Molar ratio' $= \dfrac{\text{Increase in } Q_{O_2} \times 176}{\text{Corrected loss of ascorbate} \times 22{\cdot}4}$.

assumption. In correlating these results it was necessary to take into consideration two established facts, firstly, that the loss of the ascorbate was proportional to the weight of the tissue employed and secondly that a loss of ascorbate was also incurred, even when tissue from animals on a normal diet was used, although no increase in Q_{O_2} took place. The mean relative loss of ascorbate calculated as μg. of ascorbic acid per mg. of dry liver tissue, from 21 normal animals, the respiring tissues of which showed no increase in Q_{O_2}, was 3. The observed relative loss in the case of the tissues from restricted animals was therefore corrected by subtracting this value from it. This correction was particularly justified by the fact that the relative loss of ascorbic acid in the respiration of tissues from restricted animals which showed a small increase in Q_{O_2} was not very much higher than that from normal animals. The molar ratios of (increased O_2 uptake due to ascorbate/corrected loss of ascorbate) thus obtained will be seen from Table 10 to be of the order of 3–4, which is in excess of the relative amount of O_2 required to oxidize ascorbic acid to dehydroascorbic acid ($\frac{1}{2}$ mol. O_2 per mol. ascorbic acid) or even to oxalic and *l*-threonic acids (1 mol. O_2 per 1 mol. ascorbic acid). As there was no proportional increase in the CO_2 formation (Table 8) it is hardly likely that the oxidation of the ascorbic acid proceeded to complete breakdown to CO_2 and water, which would produce 6 mol. CO_2 to 5 mol. O_2 per mol. of ascorbic acid. Even when low concentrations of ascorbate such as $10^{-4}M$ were used the molar ratio was of the same order as in the case of higher concentrations. The results rather suggest that the increase in Q_{O_2} is not due to the oxidation of the added ascorbic acid *per se* but that the compound acts catalytically on the respiration of liver tissue from the dietetically restricted animals, but does not usually do so on that of tissues from normal animals. The observed loss of the ascorbic acid is in all probability incurred whilst the acid is passing through the labile stage as dehydroascorbic acid. The observation that the greater the increase in respiration, the greater the destruction of the added vitamin (Table 10) might according to this view have been expected. It is to be noted that the loss of the ascorbic acid analogues

when added to respiring liver slices from animals on a restricted diet was of a similar magnitude to the loss of ascorbate.

There is no evidence so far to show whether ascorbic acid also stimulates the respiration of the liver of the intact guinea-pig on a restricted diet. The gas metabolism of scorbutic guinea-pigs, but not of guinea-pigs on a deficient diet containing vitamin C, has been studied by a number of workers and their results are contradictory. Abderhalden [1921] maintains that there is no difference between respiration of normal and scorbutic guinea-pigs. Jarussowa [1926], Soderström & Törnbloom [1933] and Scoz *et al.* [1937] found that the O_2 consumption of scorbutic guinea-pigs was less than that of healthy animals, whilst Calcinai & Galigani [1934] and Mosonyi & Rigó [1933] assert the contrary. Baucke [1938] concludes that the O_2 uptake of guinea-pigs on a scorbutic diet fluctuates greatly from day to day. The stimulating action of ascorbic acid on the liver respiration of underfed guinea-pigs *in vivo* can with our present knowledge be viewed only as a theoretical possibility. The observations made *in vitro*, nevertheless, lay bare some potentialities for future research.

As a preliminary effort an attempt was made in this investigation to ascertain whether a connexion exists between the above phenomenon and some of the links in schemes of carbohydrate metabolism. The influence of inhibitors, known to interfere with some of the stages postulated to occur in these schemes, was accordingly investigated on the accelerating action produced by added ascorbic acid on the respiration of liver slices from dietetically restricted animals as well as on that of liver slices from normal animals.

The failure of cyanide to influence the relative increase of the O_2 uptake produced by the addition of ascorbate in liver slices from animals on a restricted diet, excludes the possibility of this reaction being associated with the ultimate stages of the aerobic oxidation of carbohydrates. The great reduction in Q_{O_2} increase brought about by the action of phloridzin, which is known to inhibit the phosphorylation of carbohydrates, the first stage in the breakdown of glycogen, suggests a possible connexion of the accelerated respiration produced by ascorbate in the liver tissue from underfed animals with any one of the subsequent stages in the degradation of this carbohydrate. It does not however throw any light on the actual stage involved. The further fact that iodoacetate does not prevent the accelerating action of added ascorbate rather indicates that the increased respiration may be connected either with any of the stages concerned in the anaerobic formation of triosephosphate from glycogen or in the aerobic degradation of the intermediary products, but not with the compounds formed by the anaerobic breakdown of triosephosphate. The results obtained with fluoride point to the same conclusion but in a rather less striking manner. The ineffectiveness of malonate as an inhibitor excludes the possibility of the association of succinic dehydrogenase with the phenomenon under discussion. The results obtained with pyrophosphate and hydroxymalonate are difficult to interpret. Although pyrophosphate, which inhibits the action of succinic dehydrogenase, has also a retarding action on the accelerating effect of the added ascorbate, a connexion between the two must be ruled out in view of the results obtained with malonate. The possibility that the inhibitory action of pyrophosphate on the increase in the Q_{O_2} brought about by the addition of ascorbate is due to its precipitating effect on certain divalent catalytically active cations [Ochoa, 1939] cannot be excluded. It is unlikely that the action of hydroxymalonate has any connexion with the oxidation of lactate [cf. Jowett & Quastel, 1937] since iodoacetate which prevents the formation of lactate does not inhibit the stimulating effect of the added ascorbate. The significance of the negative

results obtained with maleate and arsenite cannot at present be interpreted. The observation that cinnamate does not influence the phenomenon under observation excludes its being connected with the oxidation of fats, or at least of crotonic and butyric acids, since Jowett & Quastel [1935] found that cinnamate inhibited their oxidation to acetoacetic acid.

The results so far obtained by us in our effort to link up the accelerating action of ascorbic acid and its analogues on the respiration of the liver tissue from dietetically restricted guinea-pigs with known reactions in animal metabolism can only be considered as pointers, and further work is now in progress.

Summary

The addition of low concentrations of *l*-ascorbic acid increased the rate of respiration of the surviving liver tissue of about 95% of guinea-pigs maintained on a quantitatively restricted diet containing vitamin C at levels more than sufficient to protect these animals from macroscopic and microscopic lesions of scurvy. Even the respiration of liver tissue from guinea-pigs on a restricted diet fully saturated in respect to vitamin C behaved in this way. The liver tissue of only about 30% of animals on a normal diet responded to the addition of *l*-ascorbic acid. The liver tissue from both growing and adult guinea-pigs reacted alike in this respect.

The addition of compounds chemically related to *l*-ascorbic acid such as *d*-glucoascorbic acid and reductic acid, which possess a reduction potential of the same order as *l*-ascorbic acid but no antiscorbutic activity, also stimulated the respiration of liver tissue from guinea-pigs on a restricted diet.

Some added *l*-ascorbic acid is lost during the process of respiration, but the loss is insufficient to explain the increase in Q_{O_2} on the assumption that this compound is oxidized to oxalic and *l*-threonic acids, nor do measurements of CO_2 production suggest complete breakdown of the acid. The loss of the added *l*-ascorbic acid analogues during respiration was of a similar magnitude to that of *l*-ascorbic acid. At low concentrations increasing amounts of *l*-ascorbic acid produced a more pronounced increase in respiration than at higher levels, until a level was reached when further addition ceased to influence the rate of respiration.

The increase in respiration was largely abolished by phloridzin, pyrophosphate and hydroxymalonate; it was not affected by maleate, arsenite, malonate or cinnamate. Iodoacetate depressed the Q_{O_2} value of liver tissues from normal animals but the addition of *l*-ascorbic acid prevented this inhibition. In the case of animals on a restricted diet the simultaneous addition of iodoacetate and *l*-ascorbic acid brought about a higher O_2 uptake than the addition of *l*-ascorbic acid alone. Fluoride decreased the respiration of the liver tissues from animals on a restricted diet in the absence, but not in the presence, of *l*-ascorbic acid. The relative increase in Q_{O_2} produced by added *l*-ascorbic acid in slices from animals on a restricted diet was the same in the presence of cyanide as in its absence, in spite of the great reduction in respiration produced by this reagent. The bearing of these results on the possible connexion between the accelerating effect of added *l*-ascorbic acid on the respiration of liver tissue from guinea-pigs on a restricted diet and their carbohydrate metabolism is discussed.

We are indebted to the Medical Research Council for whole time grants to A. E. K. (Pt I) and G. A. S. (Pt II). Our thanks are also due to Prof. E. L. Hirst for placing at our disposal a specimen of *d*-glucoascorbic acid and to Messrs Roche Products for a gift of *l*-ascorbic acid. Finally we should like to take this

opportunity of expressing our gratitude to Dr R. G. Hatton, Director of East Malling Research Station, for laboratory accommodation during the final part of this investigation.

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86. PROTEIN PRODUCTION BY NITROGEN-FIXING BACTERIA UNDER CONDITIONS OF CONTINUOUS AERATION

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(*Received 25 June 1941*)

Since Beijerinck [1901] isolated two species of *Azotobacter* and proved them to be capable of fixing atmospheric nitrogen, many workers have studied this group of organisms and the influence of different factors on their rate of growth and of nitrogen fixation. A suitable medium, such as that used by Ashby [1907], is one containing mannitol and inorganic salts, but no source of nitrogen. The aeration essential for rapid growth is usually ensured by cultivating the organisms in a shallow layer of the medium with a large exposed surface, but good growth also occurs in deeper layers if the medium is aerated by bubbling air through it during the course of incubation. Thus Deo [1920] inoculated such a medium with activated sewage sludge and compared the nitrogen fixation in cultures through which a current of air was passed at room temperature with that in cultures incubated in the quiescent condition at 35°. Fixation of nitrogen during 2–5 weeks was appreciable under both these conditions but the cultures aerated at room temperature gave the higher yield.

The experiments of Ardern & Lockett [1914], who, in collaboration with Fowler, developed the activated sludge process of treatment of sewage, showed that continuous aeration of sewage with a rapid stream of air resulted after some time in the deposition of a sludge containing much of the organic matter, largely in the form of bacterial cells. On allowing the sludge to settle and adding to it a fresh batch of sewage a second period of aeration resulted in the formation of more sludge and a more rapid clarification of the sewage than had occurred previously. Repetition of this process resulted eventually in the formation of a considerable quantity of 'activated' sludge which then clarified sewage, with which it was aerated, in a few hours, compared with the period of several weeks which had been found necessary in the first instance.

It seemed likely that this principle might be applied to a suitable medium containing nitrogen-fixing bacteria, the object being to obtain continuous fixation of nitrogen with the periodic removal in the form of sludge of the bacterial protein which had been formed.

Experimental

Mannitol medium

Preliminary experiments were made with a N-free solution of the following composition: mannitol 20 g.; K_2HPO_4 0·2 g., $MgSO_4$ 0·2 g., NaCl 0·2 g., $CaSO_4$ 0·1 g., $FeCl_3$ 0·006 g., water 1000 ml. The solution was neutralized with NaOH to phenolphthalein and was not sterilized.

A Winchester quart bottle, containing 2 l. of this solution, was fitted with a rubber bung and glass leading tubes to enable a current of air to be passed

continuously through the liquid by suction from a water pump. The lower end of the inlet tube was blown into the form of a bulb perforated with pin holes to break up the current of air into small bubbles. By interposing a tap between the suction pump and the bottle and connecting the inlet tube to a rotameter the flow of air was adjusted and maintained, in this and in subsequent experiments, at 5 cu. ft. an hour. The bottle was immersed in a thermostat at 25°.

In order to prevent developed acidity from inhibiting the growth of N-fixing bacteria, 10 g. of $CaCO_3$ were added to the solution. The inoculum consisted of 25 ml. of thick activated sludge produced in the laboratory from domestic sewage. This provided the source of N-fixing organisms and probably acted also as a nucleus for the formation of more sludge.

The N content of the mixed suspension was determined, using the semi-micro-Kjeldahl method described by Rowland [1938], on duplicate quantities of 25 ml., and the level of the remaining liquid was marked on the bottle. Aeration was then begun and at suitable intervals the liquid lost by evaporation was replaced by addition of water; further samples were then abstracted for determination of nitrogen. A preliminary experiment, in which the liquid was aerated for 18 days, showed that the N content increased from 2·53 to 9·46 mg./100 ml.

In the second experiment samples were taken more frequently to measure the rate at which N fixation occurred and to find how long was required in each period of aeration to reach the maximum. During the first period of aeration the N content increased from 2·95 to 10·28 mg./100 ml. in 7 days. After 5 more days there was no further increase; in fact there was a slight decrease. On the 8th day 100 ml. of the liquid were poured into a measuring cylinder and allowed to settle. The chalk settled quickly and was soon surmounted by some lighter, cream-coloured floccules. The supernatant liquid was dark brown, very turbid and showed no signs of clarifying. After 12 days' aeration the colour of the liquid was brownish black and the suspended matter still did not settle readily. Microscopic examination of the supernatant liquor showed an apparently almost pure culture of a large, oval or round organism, while dilutions of the liquid plated on mannitol-agar gave rise to a predominance of large, colourless, mucoid, surface colonies. These observations suggested that the organisms responsible for fixation of N in the aerated liquid were species of *Azotobacter*. Since the amount of activated sludge originally added as an inoculum was insufficient to colour the liquid appreciably, the dark brown colour after several days' aeration was probably due to *Azotobacter* pigment. Other workers (e.g. Ashby [1907]) have noted the appearance of a dark brown colour in ageing cultures of this organism.

On the 13th day the aeration was stopped and the mixture was left overnight. A greyish white sludge appeared at the bottom, beneath an opaque, dark brown liquor. This supernatant liquor contained a large proportion of the total N of the culture. Its N content was 6·59 mg./100 ml., compared with 10·05 mg./100 ml. in the whole culture. The NH_3 content of the supernatant liquor was extremely small so that most of the N presumably represented proteins or their decomposition products. Centrifuging for 30 min. at 2000 r.p.m. did not clarify the liquor and caused the separation of only a small amount of brown deposit.

The greyish white sludge which had settled at the bottom of the mixture, and the deposit obtained after centrifuging the supernatant liquor, were transferred to a second Winchester bottle, a fresh quantity of mannitol medium was added, and a second period of aeration commenced. The N content then increased from 5·47 to 16·27 mg./100 ml. in 5 days. After 7 days aeration was stopped. The liquor was now light brown in colour and the sludge separated on centri-

fuging more easily than at the end of the first aeration. The settled sludge and the deposit after centrifuging were again transferred to a fresh bottle of mannitol medium and a third period of aeration was begun. The N content on this occasion increased in 6 days from 12·63 to 18·88 mg./100 ml. The rate of fixation was slower than in the previous aeration and the amount of N fixed was appreciably less. The liquor and sludge at the end of the period were light coffee colour.

The figures for N content of the liquid at various stages in each of the periods of aeration are shown in Table 1. The experiments showed that considerable

Table 1. *Nitrogen fixation in aerated mannitol solution*

(mg. N/100 ml. culture)

Time (days)	0	1	2	4	5	6	7	12	13
1st Aeration	2·95	—	—	—	—	—	10·28	10·05	Stop
2nd Aeration	5·47	9·04	11·33	14·23	16·27	—	Stop	—	—
3rd Aeration	12·63	13·93	16·39	18·63	—	18·88	—	—	—

fixation of N occurred under the conditions described and that the deposit or sludge formed during one period of aeration could be collected and added to a fresh batch of medium in order to carry on the process in the next period of aeration. If the process is to be satisfactory, however, conditions must be found in which the sludge settles easily so that the supernatant liquor can be siphoned away, and the N lost in the discarded fluid must be relatively small.

Glucose medium

A similar series of experiments was carried out in which the nutrient medium contained glucose instead of mannitol but was otherwise of the same composition as that given previously.

The figures for N content of the liquid at various stages during four successive aerations are shown in Table 2. It may be seen that there is a marked lag phase

Table 2. *Nitrogen fixation in aerated glucose solution*

(mg. N/100 ml. culture)

Time (days)	0	1	2	3	4	5	6	7	8	9	10	12
1st Aeration	2·43	—	—	2·68	—	5·09	—	—	—	14·61	—	Stop
2nd Aeration	6·54	9·38	13·91	—	20·41	—	20·06	—	Stop	—	—	—
3rd Aeration	14·90	16·49	18·92	20·30	—	22·95	—	24·64	—	—	Stop	—
4th Aeration	5·58	—	10·77	—	13·36	—	18·58	—	19·04	—	—	—

at the beginning of the first aeration, corresponding presumably to the time required to establish active growth of the relatively small number of N-fixing bacteria present in the inoculum of sewage sludge. In the subsequent aerations, when there are large numbers of N-fixing bacteria at the commencement, there is no lag phase.

During the first period of aeration colour changes in the medium were observed which were similar to those noted in the experiments with the mannitol medium. For several days the deposit was white or cream in colour, and the supernatant liquid was colourless. Towards the end of the aeration period of 12 days the colour darkened and was finally very dark brown. The sludge deposited on allowing the mixture to stand and the deposit remaining after centrifuging the supernatant fluid were transferred to a fresh bottle of glucose medium for the second period of aeration.

During the second and subsequent aerations this dark colour no longer appeared and the sludge produced was light coffee colour. Moreover, at the end of the second period of aeration the sludge settled quickly, leaving a supernatant liquid which was colourless and almost clear. Thus from 100 ml. of the mixed liquor, which was poured into a measuring cylinder, the sludge which settled occupied 50 ml. after 15 min. and 33 ml. after $2\frac{1}{4}$ hr. The N content of the supernatant liquor was small, being only 2·36 mg./100 ml. when that of the mixed liquid was 20·06 mg.

Microscopic examination of the sludge at this point showed the presence of numerous large round organisms, corresponding in appearance with that of *Azotobacter*, together with considerable numbers of other bacteria, mainly rod forms. When the liquid was plated on mannitol-agar large mucoid surface colonies developed but were accompanied by a large number of much smaller colonies. It is well known that, although a mannitol medium encourages an almost pure culture of *Azotobacter*, the corresponding glucose medium allows the growth of other organisms to a considerable extent. Since many of these contaminants occurred in chains and tangled groups it is possible that they assisted in floccule formation by acting as binding agents for masses of *Azotobacter* cells, thus facilitating the settling of the sludge.

The contents of the bottle were allowed to settle for $1\frac{1}{2}$ hr. at the end of the second aeration, the supernatant liquor was siphoned off and replaced by a fresh batch of glucose medium for the next aeration. It may be seen from Table 2 that during the third period of aeration the rate of N fixation was lower than during the second aeration. At the end of this period the mixture did not settle easily and there was no clear demarcation between sludge and supernatant liquor after several hours. Since the initial N content had been high and the sludge now occupied an appreciable proportion of the total volume of the mixture it seemed likely that ability to settle, and possibly rate of N fixation, became less when the concentration of sludge protein passed a certain point. This possibility was confirmed when, on taking one quarter of the contents of the bottle and transferring it to a fresh bottle of glucose medium for the fourth aeration, it was found that N fixation proceeded rapidly, as can be seen from the figures in Table 2. After 4 days' aeration, when the N content was 13·36 mg./100 ml., the sludge settled rapidly from the standing liquid and left a fairly clear supernatant fluid. After a further 2 days' aeration, when the N content had risen to 18·58 mg./100 ml., the sludge settled very slowly indeed. It was noticed that mixtures containing a high proportion of sludge were much more viscous than those with a lower proportion and the slowness of settling may have been due to this.

These results suggest that in order to end each aeration period with the sludge in a suitable condition for rapid settling, the amount of sludge carried over from the previous aeration should be sufficiently small to ensure that the N content of the liquid does not rise above the critical value towards the end of the period of aeration. On the other hand the quantity of sludge added at the beginning of the aeration should be sufficient to allow of rapid N fixation without a lag period. From the figures in Table 2 it appears that the optimum conditions will be obtained if, after allowing the sludge to settle at the end of one period of aeration, sufficient is added to the next batch of medium to give an initial N content of 5–6 mg./100 ml. of mixture. The residue of sludge not used for carrying on the next aeration can be removed to build up a stock of protein formed by fixation.

Conclusions

1. When a nitrogen-free solution, containing mannitol or glucose, inorganic salts and sufficient chalk to neutralize acidity, is inoculated with activated sewage sludge and aerated with a rapid current of air, active fixation of nitrogen occurs through the growth of nitrogen-fixing bacteria. The deposit of bacterial sludge which separates on allowing the liquid to stand, may be transferred at the end of one aeration to a fresh batch of medium for the next aeration. In this way the process becomes continuous.

2. The sludge settles quickly and completely from a glucose medium under suitable conditions, leaving a comparatively clear supernatant liquid, in which the nitrogen content is low. If, however, the concentration of sludge in the mixture is allowed to rise above a critical value, settling of the sludge floccules becomes very sluggish, possibly owing to the increased viscosity of the liquid.

3. The rate of nitrogen fixation appears to be highest when the conditions are most suitable for the settling of the sludge.

4. Under optimum conditions in these experiments approximately 7 mg. of nitrogen were fixed for every gram of glucose utilized. It is possible that, by improving the conditions of aeration and experimenting with the constituents of the medium, both the rate and total amount of nitrogen fixation might be increased.

5. The method may be used to build up a stock of bacterial protein accompanied by the other constituents of the bacterial cells.

The experiments described in this paper were carried out as part of the programme of the Water Pollution Research Board of the Department of Scientific and Industrial Research and the results are published by permission of the Department.

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87. VITAMIN A IN BLOOD AND ITS RELATION TO BODY RESERVES

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(*Received 11 June 1941*)

An important line of vitamin research during recent years has been the establishment of chemical tests for the detection of vitamin deficiencies in human beings. While the presence of clinical signs will indicate the existence of a gross deficiency, it is recognized that the criteria for the detection of a partial deficiency are difficult to define.

A number of studies have been made with the object of correlating the level of vitamin A in blood with the state of vitamin A nutrition. Some evidence has been obtained that the level of vitamin A in blood is in general related to the economic status of the subject [Menken, 1932, 1934; Wolff, 1936; Wolff *et al.* 1936; de Haas & Meulemans, 1938, 1]. The subjects from the low-income groups were presumed to possess inadequate stores of the vitamin, which in turn were supposed to be reflected in the 'low' vitamin A values in blood. Arbitrary standards were suggested, describing certain levels of blood values as 'satisfactory' and others as 'insufficient'.

Also, it was found that generally the blood values of subjects with xerophthalmia contained either little or no vitamin A [Sie, 1937; de Haas & Meulemans, 1938, 2]. This finding appeared to fit in with the theory that the vitamin A level in blood is an index of the state of vitamin A nutrition. De Haas & Meulemans [1938, 2], however, also observed that at least 4 out of the 27 subjects with xerophthalmia had what the authors regarded as 'relatively high' vitamin A values in their blood and no explanation for this finding could be suggested.

Studies carried out by van Veen [1937] have shown that very often a direct relation between vitamin A deficiency and the vitamin A level in the blood appeared to be absent. What were regarded as 'high' or 'normal' values were sometimes found in patients with clinical symptoms of vitamin A deficiency, while 'low' values were observed in apparently healthy individuals. It appeared to us that such apparent anomalies can only be explained by the assumption that the level of blood vitamin A is not related to the body stores.

In all past work on this subject, no details of the previous dietary intake of the vitamin appear to have been taken into consideration. Without such data, the state of vitamin A nutrition of a subject is not definitely known. Thus it may happen that the diet of a subject at the time of the examination is deficient in some food factor and yet his previous diet may have provided him with ample reserves of that factor.

It appeared that the relation of the level of vitamin A in blood to the amount in the body reserves has not been defined and the present study was planned to throw more light on it. It was felt that the first step was to work on experimental animals whose whole dietary history was known and whose vitamin A intake could be easily kept under control.

The rat was found to be unsuitable for such studies because its blood level of vitamin A is normally too low to permit a reliable estimation to be made by the $SbCl_3$ reaction. Subsequently dogs were chosen for this study. After this work was started there appeared a paper by Steininger *et al.* [1939] on the blood vitamin A level of human beings whose vitamin A intake was controlled for a period of about 5 months. Although the amount of vitamin A in their fasting blood was, on the whole, found to be dependent on the amount in the diet, the evidence as to whether or not such determinations are of value in judging the nutritional status was considered by the authors to be contradictory.

Experimental

Study limited to vitamin A. Vitamin A metabolism in the animal body is intimately connected with the utilization of the various isomers of carotene. At present little is known of the quantitative aspects of carotene metabolism. It is very probable that the extent of its absorption, storage and transformation into vitamin A *in vivo* is governed by many factors which are as yet unknown. In order to reduce the problem to its simplest form it was decided that the experiment should be first carried out with reference to vitamin A alone. In this way the interpretation of the results would be simplified, since the evaluation of the significance of the carotenoid level in blood would not arise. With this consideration in view, the dogs were kept on a basal diet which was practically free from carotenoid pigments and a vitamin A concentrate (B.D.H.) was used as a supplement.

Plan of the experiment. The plan of this study was to rear young puppies of about 6 weeks on a diet of known vitamin A content. When about 6 months old, they were transferred to a vitamin A-deficient diet which was supplemented with graded doses of vitamin A. Their relative, though not absolute, reserves of vitamin A are thus known. The levels of vitamin A in their blood were determined at regular intervals to see if they were related to the vitamin A reserves. The object was to discover whether the vitamin A level in the blood can be used as a means of detecting inadequate vitamin A reserves.

This study can be conveniently divided into 3 parts: (1) a preliminary period of about 4 months during which an adequate diet of known vitamin A content was given to the puppies; (2) a dosing period of about $2\frac{1}{2}$ months during which the animals received graded doses of vitamin A daily and the vitamin A in their fasting blood was determined at regular intervals; (3) a depletion period of about 16 months on a vitamin A-free diet. In the first 6 months the blood of these dogs was also assayed regularly for vitamin A. The dogs were weighed periodically and signs of vitamin A deficiency were watched for during the depletion period.

Diet during the preliminary period. The puppies were placed on the following diet when about 6 weeks old: raw (slightly polished) rice 500 parts, skimmed milk powder 100 parts, marmite 10 parts and coconut oil 20 parts. The rice was cooked before the other ingredients were added and 2 meals (morning and evening) were given daily. The skimmed milk powder was found by chemical tests to contain about 1·0 I.U. of vitamin A per g. Each dog in addition received 2 ml. of cod liver oil (180 I.U. vitamin A) daily and 50 I.U. vitamin D daily in the form of a diluted solution of Ostelin (Glaxo).

Diet during the dosing period. This was similar to that given above with the exception that the skimmed milk powder was extracted twice with hot 95% alcohol. The skimmed milk powder (500 g.) was heated in shallow dishes in an air oven for 12 hr. at 110° and was then extracted with 2 l. EtOH under a reflux

condenser for 8 hr. After filtration by suction the milk powder was extracted again in a similar manner, after which it was filtered off and dried. That this basal diet is reasonably free from vitamin A is shown by the observation that rats kept on it developed xerophthalmia in about 6 weeks.

Vitamin A supplements during the dosing period. At first 4 dogs were given the following amounts of vitamin A: 0, 100, 400 and 2000 I.U./kg. body-weight per day. Unfortunately the negative control animal died under anaesthesia soon after the experiment was started. The vitamin A supplement was given in the form of a concentrate (B.D.H.) diluted in olive oil, and its potency was checked periodically by means of the $SbCl_3$ reaction. The required amount of vitamin A solution was mixed with the morning meal and precautions were taken to ensure that the supplement was consumed. It was observed that the dogs invariably ate their morning meal, with the exception of the dog on the highest level of dosage (2000 I.U./kg. body-weight per day) which began to lose its appetite about 8 weeks after the dosing started. This loss of appetite was accompanied by a loss of weight and was presumably due to the excessive intake of the vitamin since the appetite returned and the weight again increased when the supplement was discontinued.

Diet during the depletion period. During this period of about 16 months the vitamin A supplements were stopped and the dogs were kept on the vitamin A-free diet mentioned above.

Total intake of vitamin A by the dogs. This was calculated by taking into consideration the vitamin A administered in the form of cod liver oil during the preliminary period and in the form of the concentrate during the dosing period. The following table summarizes the figures.

Table 1. *Total intake of vitamin A by the dogs*

	Preliminary period I.U.	Dosing period I.U.	Total I.U.
Dog A	26,000	56,000	82,000
Dog B	26,000	198,000	224,000
Dog C	26,000	1,064,000	1,090,000

As these figures represent almost all the vitamin A received by the dogs (with the exception of that obtained during their first 6 weeks of life), it is reasonable to conclude that their relative stores of vitamin A were of the same order of magnitude as their vitamin A intake.

Determination of vitamin A. Blood was drawn from the dogs once in about 3 weeks during the dosing period. The dogs were anaesthetized with a mixture of equal parts of ether and $CHCl_3$ and 15 to 30 ml. were taken from a vein in the outer part of the hind leg. Blood was drawn in the morning at least 24 hr. after the previous dose of vitamin A had been consumed. The vitamin was estimated as follows. The blood was diluted with an equal volume of water in a flask and 10 ml. each of 95% EtOH and 50% aqueous KOH added. The mixture was heated in an air oven at 100° for 45 min. and then extracted with 3 successive lots of ether (100, 100 and 50 ml. respectively). The combined ether extracts were washed with water until free from alkali and evaporated to dryness *in vacuo*. The residue was then taken up in a known volume of chloroform and the vitamin A estimated by the $SbCl_3$ reaction in a Lovibond Tintometer. The blue units (Moore) were converted into I.U. by multiplying by the factor 0·6 [Moore, 1937], but in the interpretation of results these values should not be regarded as absolute but as relative only.

Results

Vitamin A in blood during the period of dosing. The results are shown in Fig. 1. It will be noted that the levels of vitamin A in the blood rose gradually throughout the period of dosing and were proportional to the vitamin A intake of the dogs. If it be assumed that their body stores were in proportion to their intakes, it can be said that the blood vitamin A level was proportional to the

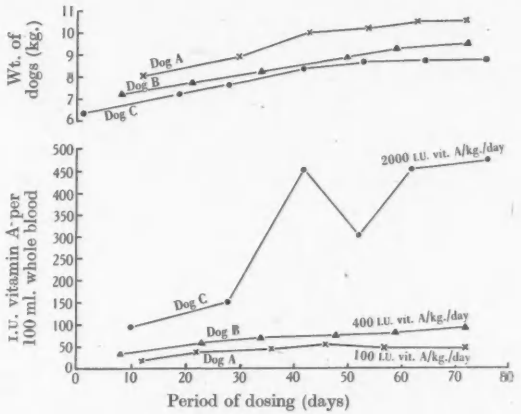

Fig. 1. Vitamin A in blood during the period of dosing. Arrow indicates commencement of dosing.

extent of the body reserves. This conclusion is valid only under certain conditions, viz. when appreciable amounts of the vitamin are present in the blood. Why this conclusion is sometimes not valid is explained below.

Vitamin A in blood during the depletion period. As soon as the vitamin A supplements were stopped the level of vitamin A in the blood began to fall (see Fig. 2). After about 6 months on the vitamin A-free diet, no vitamin A could be detected in as much as 30 ml. of blood by the $SbCl_3$ reaction (i.e. there was less than 5 I.U./100 ml. blood). There was no great difference in the time taken for the blood vitamin A of the 3 dogs to fall to 'zero' level, the respective times for dogs A, B and C being 154, 138 and 168 days.

After the blood vitamin A had fallen to this low level, the experiment was continued for another 10 months. During this period the animals were watched for signs of vitamin A deficiency but none were observed. Throughout the depletion period the dogs maintained their weights, and their general health and condition were excellent.

The female dog gave birth to a live litter of 5 young on the 276th day of the depletion period. The puppies appeared to be growing normally for about 3 weeks after which the mother's milk began to fail. The puppies quickly lost weight and 3 died while the remaining 2 were killed by coal gas. Their livers were examined for vitamin A but no appreciable amount could be detected by the $SbCl_3$ reaction.

After about 400 days on the depletion diet blood was again drawn from the dogs and examined for vitamin A, this time by the spectrophotometric method. Again no vitamin A could be detected in 30 ml. samples.

As has been mentioned above, the blood vitamin A level dropped to values of less than 5 I.U./100 ml. blood about 6 months after the vitamin A supplements were stopped. This finding, by itself, might be wrongly taken to mean that a state of deficiency existed. The important point to note is that these dogs were in a healthy condition and were free from any sign of vitamin A deficiency for a further period of 10 months.

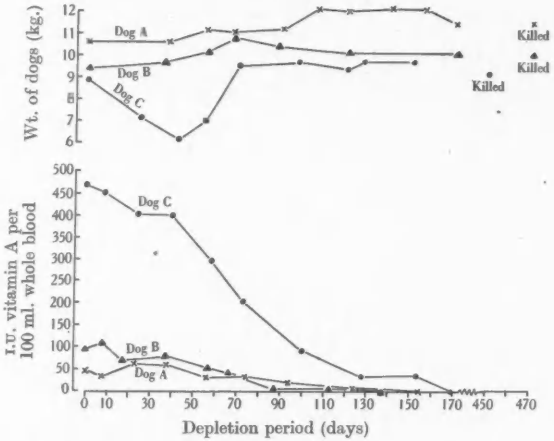

Fig. 2. Vitamin A in blood during the period of depletion.

Dog A received 100 I.U. vitamin A/kg./day for 72 days.
Dog B received 400 I.U. vitamin A/kg./day for 72 days.
Dog C received 2000 I.U. vitamin A/kg./day for 76 days.

At present the metabolic requirement of vitamin A by the animal organism is not known with any degree of certainty. According to the findings of Guilbert *et al.* [1940] the daily minimum requirement of many mammalian species is about 20 I.U. of vitamin A per kg. body-weight per day. Taking this figure as a rough guide, it can be calculated that the minimum amount of vitamin A utilized by each dog during the last 10 months of the experiment would be about 54,000 I.U. At least this amount of vitamin A was presumably stored in the dog's tissues when the level of vitamin A in its blood was practically zero. On the basis of this observation it seems that a 'low' or 'negative' vitamin A value in dog's blood (as determined by the $SbCl_3$ reaction) cannot be regarded as a sign that the vitamin A reserve is seriously depleted.

Dogs A, B and C were killed after 470, 470 and 452 days respectively on the depletion diet. No abnormalities were observed on post-mortem examination. It has been shown that distinct lesions appear in certain nervous tissues of animals suffering from vitamin A deficiency [Mellanby, 1935]. Tissues from dogs B and C were examined for histological changes by Helen F. Burn at the Department of Pathology, Singapore. The results are given in the appendix at the end of this paper. The total liver reserves of vitamin A were 70, 50 and 1370 I.U. in dogs A, B and C respectively. It appears from these figures that dogs A and B were almost completely depleted of vitamin A after about 16 months on the deficient diet.

Our results indicate that it is not possible to lay down a 'normal' vitamin A value for dog's blood. On theoretical grounds it seems reasonable to believe that there is a critical level of blood vitamin A below which signs of deficiency will appear. This level may be defined as the least amount of vitamin A in blood that is sufficient to meet the normal requirement of the tissue cells. Amounts below this level will correspond to a state of deficiency of the vitamin and the prolonged effect of this condition will result in the appearance of anatomical changes characteristic of the deficiency. It appears that the $SbCl_3$ reaction is not sufficiently sensitive to detect the critical level of vitamin A in dog's blood. It can also be concluded that there is no danger of avitaminosis-A in dogs so long as there are even traces of vitamin A in the fasting blood.

There is evidence that the critical levels of blood vitamin A in various species are different. Thus, the recent observations of Davis & Madsen [1941] indicate that the critical level of carotene in cattle plasma is comparatively high, viz. 25 μg./100 ml. and for vitamin A in the same sample about 16 μg./100 ml. When the critical level in a species is comparatively high, its estimation by the ordinary methods of vitamin A analysis will be possible and the detection of depleted vitamin A reserves can be made by blood analysis.

In the living body different levels of blood vitamin A may be found, depending on factors such as (1) the extent of the vitamin A intake and (2) whether or not the blood was drawn during the process of intestinal absorption. When the body reserves are totally depleted, it is to be expected that the blood will not contain any detectable amounts of vitamin A. On the other hand, the results of our experiments with dogs indicate that a negative $SbCl_3$ reaction does not necessarily mean that the body reserves are depleted.

APPENDIX

By Helen F. Burn

Immediately after death, the following tissues were removed from each dog: (1) fundus of the stomach, tongue and striated muscle and (2) peripheral nerve, optic nerve, spinal cord (cervical, thoracic and lumbar levels), trigeminal nerve, frontal cortex, motor cortex, basal and caudate nuclei, medulla, pons and cerebellum.

The stomach, tongue and muscle were stained with (1) haematoxylin and eosin and (2) haematoxylin and Van Gieson. They were found normal in both dogs.

The peripheral nerve, optic nerve and trigeminal nerve were studied by the Marchi and Weigert-Pal methods. The peripheral and trigeminal nerves were normal. The optic nerve from dog B showed slight degeneration in the Marchi sections.

The spinal cord and brain sections were studied by the Weigert-Pal method and also by the routine stains of (1) haematoxylin and eosin and (2) haematoxylin and Van Gieson. No change was noted except for a very slight degeneration of the lumbar cord in the posterior column in dog B. This change was probably not pathologically significant.

Summary

A study has been made of the level of vitamin A in the blood of dogs receiving graded doses of the vitamin. The experiment included a period of dosing with vitamin A and a depletion period on a vitamin A-free diet.

During the period of dosing, the level of vitamin A in the blood was proportional to the intake of the vitamin.

During the depletion period, the blood vitamin A gradually fell to a very low level (not detectable by the $SbCl_3$ reaction in 30 ml. blood).

A 'low' blood vitamin level, however, cannot be considered as a definite sign of depleted reserves of the vitamin.

I wish to thank Dr C. J. Oliveiro for his help in taking blood samples for these experiments.

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88. BLOOD COPPER OF EWES DURING PREGNANCY

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(*Received 19 June 1941*)

In a previous communication [Eden, 1941] comparison was made in connexion with an experiment on 'border pine' between the blood Cu levels in two different years of a group of sheep grazing on a Northumbrian hillside. The first samples were taken at a stage of late pregnancy in February and March 1939 and the corresponding animals were again bled in June 1940, about two months after lambing. No significant differences in the average Cu values at the two bleedings were found, but the further observation was made that the wide variations found between different individual sheep [Eden, 1939] also occurred within the same sheep at different times. Considering the group as a whole no evidence could be found of a difference of blood Cu level associated with pregnancy. Shearer *et al.* [1940], in studies on ewes in Derbyshire where prophylactic use of Cu during pregnancy has such a marked effect on the incidence of 'swayback' in lambs, observed that the blood Cu values of the ewes just after lambing were higher than in November and January bleedings, dates which corresponded to early and middle pregnancy. The average initial Cu level in their records, however, was considerably lower than the average of other ewes reported in the literature, and the authors, without giving actual protocols, state that subsequent work on sheep in Cambridge did not confirm the suggested rise during pregnancy. Beck [1940], in a private communication from Australia, also stated that he had not observed any significant rise during pregnancy in the sheep.

Several groups of workers have reported a gradual elevation in blood Cu levels of women during pregnancy. Tompsett & Anderson [1935] in a random selection of bloods of women in various stages of pregnancy reported a definite rise associated with the later stages, but unfortunately the available data on normal variations in the human subject are not sufficient to reveal the range to be expected irrespective of pregnancy. The much larger number of data accumulated for sheep over the last few years shows that extremely wide variations are encountered in apparently normal sheep and no physiological explanation for this variability has yet been offered. Equally wide normal variations may exist in other mammals, and if so they would tend to make the interpretation of considerable fluctuations of blood Cu very difficult in particular cases. It is thus probable that the securing of more data on women and increased knowledge of the normal distribution of such data might cause a revision of the idea of elevated blood Cu levels associated with pregnancy in the human subject.

The writer carried out a limited number of observations on rabbits some years ago in connexion with studies on foetal Cu and observed no rise in blood values of the does during pregnancy, but at the time it was felt that the data were insufficient to warrant a definite conclusion. More recently the increasing recognition of the importance of Cu in relation to certain diseases of sheep has stimulated further interest in the possible importance of variations in blood Cu

under different conditions, and occasion was therefore taken to reinvestigate the gestation factor on an available group of 21 ewes at this Institute by blood sampling at monthly intervals throughout the whole course of pregnancy.

Experimental

The ewes were part of the Institute flock of Cheviot-Border Leicester sheep of varying ages and were crossed by a Suffolk ram about September and October. They were wintered out of doors in a sheltered paddock and received the usual ration of hay, roots and a small allowance of concentrates. They were bled each month until after they had lambed. Blood obtained by jugular puncture was collected into Cu-free potassium oxalate and the Cu was determined by the photometric diethyldithiocarbamate method [Eden & Green, 1940]. Haemoglobin was estimated by the acid haematin method using a standardized disk. The analytical data are given in Table 1 together with the overall range of Cu values for each ewe throughout pregnancy and the average value at each bleeding.

Table 1. *Blood Cu and haemoglobin of Weybridge sheep during pregnancy*

	Blood Cu mg./100 ml.								Hb g./100 ml.						
Sheep no.	Sept. and Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	Overall range	Sept. and Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.
2	0·135	0·130	0·144	0·176	0·148	0·130	0·144	0·046	12·8	13·0	11·4	12·3	12·6	13·6	13·4
3	0·158	0·135	0·236	0·190	0·153	0·094	0·176	0·142	12·7	10·9	10·7	12·2	12·2	12·5	10·8
5	0·116	0·112	0·138	0·197	0·116	—	—	0·075	12·7	12·3	12·6	12·3	13·2	—	—
17	0·116	0·180	0·130	0·188	0·122	0·112	0·122	0·066	13·9	12·4	11·5	12·6	12·5	13·6	12·2
30	0·122	0·155	0·247	0·141	0·153	—	—	0·125	12·1	10·8	10·2	12·9	11·9	—	—
36	0·132	0·108	0·154	0·141	0·158	0·116	0·178	0·070	12·6	12·3	10·7	12·7	12·2	12·8	12·2
38	0·168	—	0·108	0·135	0·130	0·102	0·120	0·066	12·3	10·9	10·6	13·0	11·2	12·0	11·1
39	0·104	0·116	0·122	0·122	0·116	0·122	0·120	0·018	10·2	10·5	10·1	11·0	9·6	10·9	10·5
296	0·209	0·100	0·074	0·109	0·088	0·070	0·140	0·139	11·7	12·0	10·8	13·3	12·1	13·4	12·9
297	0·106	0·064	0·045	0·076	0·094	0·082	0·128	0·083	10·4	11·2	10·9	13·0	11·8	12·1	12·0
298	0·239	0·140	0·118	0·132	0·100	0·116	0·135	0·139	10·9	—	11·1	13·9	12·0	13·0	11·5
299	0·126	0·180	0·094	0·144	0·135	0·220	0·144	0·094	10·5	11·8	10·9	13·2	—	12·0	11·6
300	0·085	0·124	0·110	0·132	0·112	0·144	0·120	0·059	12·0	12·8	10·9	14·3	12·4	12·7	13·4
301	0·106	—	0·076	0·106	0·173	—	—	0·097	11·8	—	10·8	13·9	12·8	—	—
302	0·148	0·084	0·050	0·064	0·074	0·097	0·082	0·098	10·2	10·9	10·0	11·9	11·5	9·4	12·8
303	0·102	0·080	0·088	0·088	0·110	0·120	0·108	0·040	10·7	10·7	9·4	12·6	11·6	12·4	8·9
304	0·112	0·100	0·118	0·120	0·156	0·150	—	0·056	10·3	11·1	10·9	12·4	16·1	13·9	—
305	0·108	0·126	0·108	0·091	0·114	0·144	0·124	0·053	10·9	12·7	10·5	13·7	12·3	12·2	13·2
961	0·122	0·122	0·171	0·148	0·148	0·144	0·160	0·049	11·8	10·7	8·9	11·3	12·0	11·7	10·7
998	0·120	0·110	0·124	0·135	0·158	0·188	0·130	0·078	12·6	11·1	11·1	13·0	12·6	12·8	12·8
1000	0·130	0·155	0·158	0·135	0·135	0·198	0·138	0·068	12·6	10·9	10·6	13·2	12·1	13·9	13·0
General mean	0·132	0·122	0·124	0·132	0·128	0·137	0·132	—	11·7	11·5	10·7	12·8	12·2	12·5	11·9
Group mean	0·136	0·122	0·107	0·116	0·123	0·136	0·132	—	11·7	11·5	10·6	12·8	11·9	12·4	11·9

Four sheep either aborted or gave birth to dead lambs and, being required for other work in the Institute, were not bled again. Of the original 21 animals, 17 were bled all through the period of pregnancy but no samples are recorded for Nos. 5, 30, 301 and 304 after the date of abnormal parturition. In determining the average Cu values at each bleeding two means are given corresponding to (*a*) the 17 animals that went to full term and (*b*) all the animals at each bleeding. With the exception of the December and January figures these means are practically identical, but the slight differences at these bleedings are accounted for by omission of the high values of two animals, Nos. 5 and 30, from the averages. Considering the values as a whole the averages are for all practical purposes constant throughout the period of pregnancy.

Discussion

The general run of the blood Cu figures shows that the Institute sheep have higher levels (0·13 mg./100 ml.) than those reported from the Northumbrian area (0·08 mg./100 ml.) or from the Derbyshire 'swayback' area. The haemoglobin data give no indication of anaemia, all values being within the normal range for sheep, and there is no apparent correlation between Hb and Cu levels. The general mean of all the blood Cu figures in the present investigation is 0·130 mg./100 ml., a figure of the same order as the 0·117 reported in 1939 as the mean of 18 laboratory sheep selected at random irrespective of age, sex or condition. 69% of the values lie within $\pm 0{\cdot}03$ mg./100 ml. of the mean, an order of variation similar to that already reported for Northumbrian sheep in 1939 and 1940.

The data once again emphasize the wide variations existing between different sheep and in the same sheep at different bleedings. Of the 21 sheep only one, No. 39, had a range of variation over all the bleedings of less than 0·02 mg./100 ml., while 4 animals had an overall variation greater than 0·12 mg./100 ml. The extreme range of all values is from 0·045 to 0·239, which may be compared with that of 0·016 to 0·164 obtained in Northumbrian sheep in 1939 and 0·007 to 0·210 obtained in 1940. The extreme values bore no relation to the state of health of the animals.

The present data indicate that any changes in blood Cu level during pregnancy of sheep are within the normal range of variation for non-pregnant sheep and also that such changes as are observed are not related to the particular stage of pregnancy. A fall is as frequent as a rise and increase and decrease may alternate in successive months. As Shearer *et al.* [1940] suggest, it may be that a physiological rise in blood Cu during pregnancy may only occur when the initial level is too low to satisfy the demands of the foetus, but the evidence for this idea is meagre. It is apparent that the normal range of variation is such as to swamp possible physiological changes specifically associated with pregnancy.

SUMMARY

Blood Cu determinations were made on 21 breeding ewes at monthly intervals throughout the period of pregnancy. Although individual fluctuations were considerable the average values at each bleeding were very constant and there is no support for the view that pregnancy in sheep is accompanied by a physiological rise in blood Cu. Such changes as were observed fell within the normal range of variation previously found for non-pregnant sheep, and increase and decrease of individual values bore no relationship to the stage of pregnancy.

The author wishes to thank Dr H. H. Green for encouragement and interest in these investigations, and Mr C. W. Clarke for technical assistance in the course of their execution.

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89 THE LABILITY OF SKELETAL MAGNESIUM RESERVES. THE INFLUENCE OF RATES OF BONE GROWTH

By JOHN DUCKWORTH and WILLIAM GODDEN

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(*Received 1 July 1941*)

It was previously shown [Duckworth *et al.* 1940] that the skeletal Mg of the rat served, in part, as a reserve upon which the animal could draw to remedy soft tissue lack arising from acute dietary deficiency of the element. When Mg was restored to the diet of the deficient rat the skeletal Mg increased at a rate indicating that replenishment of the reserve was occurring. The present communication records attempts at modifying the magnitude and velocity of mobilization from the skeleton. The method of approach was to alter the rate of bone growth by adjustments of the Ca intake of rats receiving a diet acutely deficient in Mg.

We are not aware of any studies having been presented concerning massive liberations of Mg from the skeleton. Other studies in Mg deficiency by various workers are considered in the discussion. The present study is concerned only with Mg deficiency in the young growing animal. It is realized that the reaction of the skeleton to Mg deficiency might be different in the adult in which the skeleton and soft tissues have completed their growth.

Analytical methods

The methods used for analysis were those previously reported [Duckworth *et al.* 1940]. In most cases only total ash and Mg were determined, since it was established that there were no disturbances in the Ca and P contents of the bone ash in Mg deficiency, beyond a slight increase in the percentage of Ca, probably arising from the replacement of Mg by Ca in the bone salt crystal.

Experimental

The feeding and housing methods were those previously described. It is regretted that body-weight records were not kept regularly, but handling of the animals in the hypersensitive stage frequently induces convulsive attacks with a fatal termination. As regularity of survival was essential for the completion of the present study the omission was unavoidable.

Four diets were prepared with different Ca contents. Modification of the Ca content was achieved by altering the amount of $CaCO_3$ in the salt mixture with corresponding compensatory adjustments in the starch content of the diet.

Table 1. *Ca and P contents of Mg-deficient diets*

Diet	% Ca	% P	Ca : P
A	0·90	0·35	2·6 : 1
B	0·30	0·35	0·9 : 1
C	0·10	0·35	0·3 : 1
D	0·0001	0·35	0·0 : 1

The Mg content was less than 6 p.p.m.

Otherwise the diet was the same as that already recorded [Duckworth *et al.* 1940]. No attempt was made to eliminate from the diet more Ca than was removed from its constituents during their purification to reduce the Mg content.

Judging from the results obtained in other studies at present in progress of the percentages of ash in the bones, and also from a comparison with the records of Brown *et al.* [1932], diets A and B can be considered normal except for the deficiency of Mg. Diet C was deficient in Ca and diet D acutely deficient. Diet B was fed only to a small group of rats for the purpose of comparing the symptoms developed on this diet with those arising on the other diets. The skeletons of the rats on diet B were not analysed.

Nine litters, each of 7 hooded Lister (M and F) rats, were divided into 3 groups of 3 litters. Each group received one of the above diets, A, C or D. The rats were weaned at 23 days of age and transferred to stock diet for 2 days to accustom them to a non-milk regimen before feeding the Mg-deficient diet. At 25 days of age one rat from each litter was killed. A second rat was allowed to continue on the appropriate diet until death occurred. The remaining 5 rats were killed at different times during this survival interval, the killings being arranged to be more frequent during the early phase of the deficiency since it was expected that the greatest changes in the degree of mobilization would occur about this time. Occasionally an animal died before the scheduled time and, in such cases, an interchange was made with the surviving animal due to be killed on that day. A possible bias may have entered the experiment for this reason but it was not apparent from inspection of the results in the few cases in which interchange was necessary.

The skeleton was freed from flesh by the trypsin digestion method of Subrahmanyan *et al.* [1939]. After digestion the skeleton was divided into 4 parts: the long bones, the flat bones, the rib bones and the spinal plus caudal bones. The 4 parts were analysed separately. The paws and sternum were discarded before tryptic digestion. The teeth were also discarded, because in Mg deficiency their behaviour differs from that of the skeleton [Duckworth & Godden, 1940].

Table 2. *The influence of the Ca content of Mg-deficient diets upon the survival of rats*

Diet	Survival periods days	Mean days
A	5, 5, 5, 5, 7, 8, 8	6·1
B	7, 7, 8	7·3
C	9, 10, 11, 11, 12, 12, 13, 16, 16	12·2
D	8, 12, 14, 16, 24, 27	16·8

Results

Symptomatology. The results in Table 2 show the survival periods of rats dying spontaneously of Mg deficiency in the present and other studies in progress. The period of survival was lengthened by a reduction in the Ca intake. The results were erratic and overlapping of groups was extensive. Vasodilatation and hyperaemia of the peripheral vascular system occurred in groups A, B and C. The interval between the introduction to the diet and the onset of vasodilatation was extended and the severity of the condition was rendered less marked by lowering the Ca intakes. Vasodilatation occurred only in some of the rats receiving diet D and even then it was transitory and mild in nature. Assessment of differences is difficult in piebald rats which were used in the present studies

the albino rat is much superior for exact determination of the onset and persistence of vasodilatation. In group A, particularly, oedema of the ears was very marked at the time of vasodilatation. This condition also seemed less intense in groups with lower Ca intakes.

Mobilization of Mg from different parts of the skeleton

Mg appeared to be liberated at about the same rate from all parts of the skeleton in each group. The final percentages of Mg in the ash were slightly higher in the ribs and flat bones of group D rats than in the long bones and spine bones. In agreement with the observations of other workers it was found that the ribs and flat bones were considerably more reduced in ash content than the other parts of the skeleton in group D which was extremely deficient in Ca. Otherwise the changes observed in different fractions corresponded closely with those of the skeleton as a whole and detailed presentation of the results for the different skeletal fractions is therefore unnecessary.

Skeletal ash. The values shown in Fig. 1 for total skeletal ash are the sums of the values for the 4 skeletal fractions analysed. The results are slightly lower than those we have previously recorded for total skeletal ash, owing to the discarding of the sternum, paws and teeth. As was expected, 3 rates of skeletal growth were obtained (Fig. 1). In group A there was rapid deposition of bone ash and in

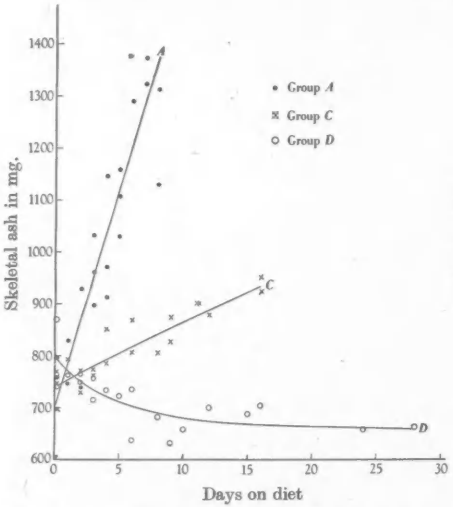

Fig. 1. The effect of different levels of Ca in Mg-deficient diets on the skeletal ash of rats.

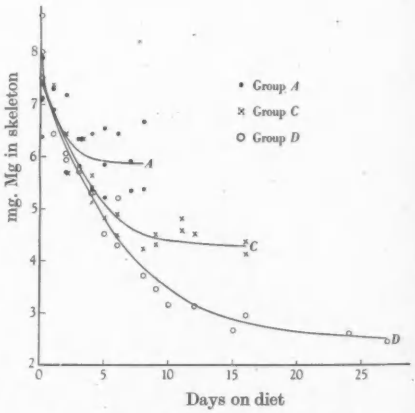

Fig. 2. The effect of different rates of bone growth on Mg liberation from the skeleton.

group C there was a much slower deposition. In group D there was a progressive demineralization of the skeleton, which proceeded more rapidly during the early part of the deficiency.

Skeletal Mg. The liberation of Mg was least in quantity and slowest in velocity in group A (Fig. 2). Considerably more Mg was liberated, and at a more rapid rate, in group C. In group D occurred the mobilization of greatest magnitude and with a rate of liberation slightly greater than that of group C. About one-

fifth of the Mg was liberated in group A rats, about one-third in group C and about two-thirds in group D. Mobilization appeared to cease in groups A and C after about 5 and 9 days respectively, but in group D, in which there was a progressive demineralization of the skeleton, the mobilization, after a rapid initial liberation, continued at a very slow rate.

The percentages of Mg in the ash fell most rapidly in the rats in group A in which bone growth was most active, and least rapidly in group C in which mineralization of the skeleton was proceeding at a slow rate (Fig. 3). In group D the curve for percentage Mg in ash coincided with that of group C for the first half of its course and thereafter assumed a path below the group C curve.

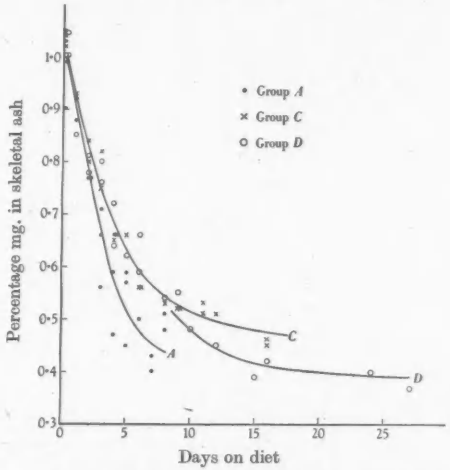

Fig. 3. The percentage of Mg in the skeletal ash of rats receiving Mg-deficient diets with different Ca intakes.

The quantities of skeletal ash and of total skeletal Mg were somewhat variable as between rats of the same age. Differences of up to 100 mg. of ash were found between newly weaned rats of the same body-weight. The percentages of Mg in the ash, especially in weaning animals, were less variable.

None of the above observations appeared to be influenced by the sex of the animal.

Discussion

It seems reasonable to believe that the survival of Mg-deficient rats will depend on two factors; first, the transport of Mg from centres where its concentration can be reduced with little or no impairment of function (e.g. bone salt) to positions where Mg is required to sustain vital processes and, secondly the adjustments that can be made in growth and metabolism to reduce the Mg requirement. Before examining the present data on the skeletal reserve in relation to the rest of the organism consideration of the distribution of Mg in the body is essential.

Of the total body water of the rat about 40% is extracellular [Harrison *et al.* 1936] and of this about one-eighth is plasma water, calculated on the basis of Skelton's [1927] data. About 30% of the plasma Mg is non-ultrafiltrable and

is thought to be in combination with plasma proteins. From this colloidal form Mg can be ionized and it thus serves as a circulatory reserve capable of partially remedying reductions in ultrafiltrable Mg. Peters [1935] considers that interstitial fluid contains only a trace of protein and that the maximum value of 0·5% is probably too high. The other fluids and transudates of the extracellular phase are generally much lower than plasma in protein content and also, in the majority of pleural and ascitic fluids of clinical cases studied by Greene *et al.* [1931], lower in Mg. It follows that, except in the case of plasma, extracellular fluid can carry but a very small reserve of protein-combined Mg. Since alterations in electrolyte levels are rapidly transmitted throughout the extracellular fluid, Mg combined with plasma protein is the main reserve of this phase, though even this is small in relation to the volume of fluid it serves and the amount which can be liberated from it is limited by the conditions of physico-chemical equilibrium obtaining.

Another possible source of Mg ions might be a combination of Mg with the proteins of interstitial tissue. The question whether Mg is bound by the organic matrix of bone is not yet settled. While the majority of workers regard Mg as mainly, if not exclusively, a bone salt component, Klement [1931] holds that it is not. Subrahmanyan *et al.* [1939] showed that when part of the organic matrix is hydrolysed by trypsin only very small quantities of Mg are lost. Further, Na, which is bound by plasma proteins, but to a less extent than Mg [Greene & Power, 1931], is not bound by any tissue except bone [Harrison *et al.* 1936] and most of this is precipitated in the bone salt [Harrison, 1937]. On the basis of the above evidence, admittedly fragmentary, it seems unlikely that interstitial tissue binds significant amounts of Mg.

Erythrocyte Mg was found by Tufts & Greenberg [1937–8, 1] to be gradually reduced in Mg deficiency, but these authors were unable to demonstrate that transport across the membrane had occurred, because of the disturbing influence of the entry into circulation of new erythrocytes low in Mg. The reduction in Mg level will, however, free Mg for use by other tissues and whether this occurs by slow transport across the erythrocyte membrane or by phagocytosis is immaterial from the present standpoint, except in connexion with the velocity of reparation of the deficit. It is generally believed that tissue cell membranes do not permit the loss of Mg, but whether this condition persists in Mg deficiency is not known.

From the foregoing it seems probable that, excluding the bone salt, the organism has very little reserve of Mg except the small amount combined with plasma protein.

Support for the foregoing evaluation of the body's Mg reserves is given by the data on the rate of liberation of skeletal Mg. The rapid release of the element from the skeleton in all groups and the absence of an initial lag suggests that there are no other reserves of significant magnitude and lability.

The large differences in the amounts of Mg liberated from the skeleton in different groups are related to differences in rates of bone growth, the mobilization increasing progressively with decreasing mineralization. During the formation of the bone salt crystal considerable interchanges of ions with those in the fluid phase of the bone occur [Logan, 1940]. While the above results show the rapidity with which Mg ions are lost by bone salt the gain of Mg ions by newly deposited bone salt is equally important. The possibility that Mg ions may even be involved in the initial precipitation as well cannot be excluded. It follows that the curves in Fig. 2 show not gross but net mobilization of Mg, that is, the quantity of Mg liberated for soft tissue use. The value obtained on analysis represents unmobilized Mg remaining in the bone salt initially present plus Mg in the salt deposited during the experimental period. An exception exists in group D

where there was a gradual reduction in the total amount of bone salt so that the analytical value represents more than the gross liberation arising from Mg deficiency, in contrast tc the state of affairs met with in groups A and C. The curve of gross liberation would lie between the curve of group D and that of group C, in which there was slight mineralization. Similarly, the velocity of mobilization, or lability, of skeletal Mg is not revealed by analysis. Only the velocity of liberation of the net quantity mobilized is obtained.

This finding that the skeleton is simultaneously a source of, and a competitor for, Mg is in agreement with our previous observations on teeth [Duckworth & Godden, 1940] where it was shown that the incisor of the rat continues to gain Mg throughout the course of the deficiency, although at a subnormal rate.

Tufts & Greenberg [1937–8, 1] state: 'the course of Mg deficiency in the rat conforms to two fairly definite phases, the first being characterized chiefly by vasodilatation, hyperaemia and hyperexcitability, and the second being marked by nutritional failure, cachexia and kidney damage'. The present observations indicate that during the first phase there are soft tissue gains of Mg from the skeleton, while the second phase persists during the period when no further exchanges occur. These workers found that plasma Mg was sharply reduced during the first phase of the deficiency and then returned to normal levels. Thereafter the level gradually declined but the previous minimum was not again reached. The interval required for the plasma Mg to reach its minimum varied directly with the Mg content of the diet. Their finding that the minimum was reached on the third day of deficiency in rats on diets containing less than 10 p.p.m. of Mg coincides closely with the time of almost complete mobilization of skeletal Mg in group A rats. On our diet, as previously shown [Duckworth *et al.* 1940], there is diminution, and finally cessation, of growth and failure of appetite after 3 days. The interrelation between exhaustion of the skeletal Mg reserve, reduction in appetite to the point of cessation of growth and the two-phase nature of the syndrome of the deficiency, is obvious.

From the data given in Figs. 2 and 3 it is clear that during the second phase of the deficiency the gains in Mg of depositing bone salt are at the expense of crystals already deposited since total skeletal Mg remains constant but the percentages of Mg gradually fall. The mechanism of exchange between the crystals is probably by gradual liberation of Mg from the older crystals in response to the slowly falling plasma Mg level. The liberation of these ions into the fluid phase of bone appears to be followed by their immediate combination with the more recently deposited crystals which are, as stated above, actively exchanging ions with the perfusing liquid during the early stages of development of the crystals. Although the percentages of Mg in bone ash ultimately reach approximately the same levels on all diets (but with consistent small differences between diets) we hesitate to suggest that the bone salt Mg exists in two forms: a labile and a bound form. The minimum values obtained indicate, it is felt, that bone salt which is being precipitated from the fluid phase of bone attracts, during its formation, a quantity of Mg proportionate to the Mg level of the fluid. That Mg ions are in any way an essential component of the crystal seems to be ruled out by the work of Logan & Taylor [1937] who obtained, *in vitro*, crystals very similar to bone salt from Mg-free solutions.

The falling level of plasma Mg has so far been considered to be responsible for the outflow of Mg ions from the bone salt. It is a point of considerable interest that, after mobilization is complete, the rise in plasma Mg is not associated with a movement of Mg back to the bone salt. As stated above, evidence has been presented to indicate an interchange of Ca for Mg ions in the

crystal during liberation of Mg. The reverse displacement of Ca ions by Mg ions when plasma Mg rises does not appear to occur. The organism appears to have no mechanism for independent liberation of Ca from bone salt. Our previous studies [Duckworth *et al.* 1940] of the effect of restitution of Mg in the diet after a period of depletion, demonstrated that Mg does not rapidly return to the skeleton. Even after 10 days of normal Mg intake the percentage of Mg in the bone ash had risen considerably but had not attained normal levels. It is tentatively suggested that the return of Mg to the depleted bone salt can only occur during redeposition following resorption. This resorption and redeposition of bone salt affords an opportunity for precipitation of crystals of normal Mg content. Such activities of the skeleton are not measurable by ordinary methods of chemical analysis.

This characteristic of Mg, that it can leave the bone salt independently and in large amounts, does not appear to be possessed by other components of the crystal, nor does it seem possible for toxic ions, such as Pb and F, to leave the crystal independently after deposition. Considering the importance of Mg as an intracellular ion this freedom of movement is probably of great value to the organism.

No mention has been made of a possible involvement of the endocrine system in the liberation of skeletal Mg. There are no records in the literature of large mobilizations, such as have been observed in the present study, after administration of hormone preparations or following the partial or complete ablation of endocrine glands. It is felt, however, that the level of Mg in the fluid phase of bone cannot alone be responsible for the removal of the ion from the bone salt crystal, since both tryptic digestion of bone and the isolation of bone salt by methods based on the complete solution of the organic matrix have little effect upon the Mg content of the bone salt. Greenberg & Mackey [1932] showed that, after parathyroid hormone administration in dogs, serum Mg increased and then returned to normal before the serum Ca elevation began, an observation we have confirmed for the horse (unpublished data). Unfortunately there has not yet been presented proof that the increment observed is of skeletal origin.

The present observations of the effect of Ca on the survival period confirm those of Tufts & Greenberg [1937–8, 1, 2]. The erratic survival periods on all diets suggest that several factors are involved. The action of normal Ca intakes in reducing the survival interval probably does not depend on the failure to liberate more than a relatively small quantity of Mg alone but also upon the so-called 'physiological antagonism' of the two elements. The death of an animal in tetanic convulsions appears to arise directly from a lack of Mg ions since administration of $MgSO_4$ solutions to animals during a seizure has only once, in our experience, failed to initiate recovery and, if the treatment were continued, survival. This is true not only in rat experiments but also in the naturally occurring Mg deficiency of milk-fed calves. Hitherto workers studying Mg deficiency have emphasized the pathological calcification which occurs throughout the body. The diets used in such experiments were, however, higher in Mg content than ours and permitted longer survivals. In the present study several rats in group A died at 5 days of age. Irving [1940], studying the rats used in the tooth experiment of Duckworth & Godden [1940], failed to detect pathological calcification in the enamel organ before the sixth day of deficiency. No other organ was examined but calcareous deposits might reasonably be expected as early in the enamel organ, with its intense Ca metabolism, as elsewhere.

SUMMARY

The net quantity of Mg liberated from the skeleton and its apparent rate of mobilization were found to be related to the rate of bone growth.

The distribution of Mg in the organism was discussed and the conclusion was drawn that the skeleton contains the body's main reserve of Mg. It was shown to be highly labile.

The two-phase nature of the syndrome of Mg deficiency was shown to be explicable on the basis of mobilization and then cessation of mobilization, of skeletal reserves.

A mechanism for the removal and return of skeletal Mg was suggested.

We desire to express our appreciation of valuable advice and criticism by Prof. J. S. Young during the course of this work.

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90. PALM OIL CAROTENOIDS

ADDENDUM TO PAPER BY HUNTER & SCOTT IN THIS *JOURNAL*, 1941, **35**, 31

By HARRY WILKINSON

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(*Received 14 June 1941*)

When Hunter & Scott [1941] published the results of their study on the lipoid pigments isolated from a 'Sherbro' palm oil they stated that the biological activities of their specimens of α- and β-carotene were in the ratio of 1 : 2. Fuller details of these tests are now submitted in this note.

The crystalline carotenes were dissolved in refined deodorized arachis oil containing 0·05% quinol to give an estimated biological potency of 500 International Units of vitamin A per g. on the assumption that 0·6 μg. β-carotene or 1·2 μg. α-carotene was equivalent to one International Unit of vitamin A.

These solutions were then assayed against the International Vitamin A Standard by the standard procedure followed in this laboratory [Morgan, 1934]. The following table gives the potencies, the number of pairs used in the assay, the limits of error ($P = 0{\cdot}99$) for the whole assay and the number of International Units per g. of crystalline compound:

	β-Carotene solution	α-Carotene solution
Estimated potency	500 I.U. per g.	500 I.U. per g.
Actual potency	600 I.U. per g.	520 I.U. per g.
Number of pairs of rats	19	21
Limits of error ($P = 0{\cdot}99$)	83–120%	76–130%
Number of I.U. per g. of crystalline compound	$2{\cdot}0 \times 10^6$	$0{\cdot}92 \times 10^6$

These results offer quite definite evidence of the fact that this preparation of α-carotene has only half the activity of the corresponding preparation of β-carotene.

If one assumes that this preparation of α-carotene is representative of α-carotene as a whole, then the results reported here confirm the finding of Kuhn & Brockmann [1933] and Kuhn *et al.* [1933] that α-carotene has half the activity of β-carotene—an observation which has never hitherto been confirmed [Hart, 1940].

The biological results with the β-carotene would suggest that the β-carotene forming the International Standard was only 90% pure—a finding confirmed by a comparison of the spectrophotometric data obtained in this Laboratory on both materials.

The author wishes to thank the Directors of Lever Brothers and Unilever Limited for their permission to publish these results.

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91. THE EXCRETION OF VITAMIN A IN URINE[1]

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(*Received 4 July 1941*)

SINCE vitamin A is insoluble in water one would not expect to find it in urine. As far as normal human urine is concerned this expectation appears to be correct. An early claim by Cooper [1924] to have detected vitamin A in human urine by biological means was criticized by Rowntree [1930], who could not confirm the presence of the vitamin in urine from children even when the diet contained milk, eggs, carrots and cod-liver oil. Kaufmann & Drigalski [1933] obtained negative results from human urine by colorimetric methods. In the urine of rats Davies & Moore [1934] found no vitamin A by the colorimetric method even when toxic overdoses of the vitamin were given. Przezdziecka [1935] claimed to have detected vitamin A in urine by a colorimetric method, which was, however, applied in so unusual a manner as to leave doubt as to the significance of the observation.

In urine from pathological subjects, however, Boller & Brunner [1936] obtained positive results by the $SbCl_3$ method in 10 out of 42 cases examined. Half of the patients excreting the vitamin had cancer. Later, Boller *et al.* [1937] published results on 321 cases. Vitamin A was found in the urine in icterus with closure of the biliary duct, chronic nephritis, nephrosis, lobar pneumonia before crisis and cirrhosis of the liver. Large oral doses of vitamin A had little effect on the amounts excreted in the urine. Excretion was reduced by pyramidone.

The presence of vitamin A in pathological urine was confirmed by Schneider & Weigand [1937, 1, 2]. Excretion was observed in cancer, tuberculosis and chronic infections. The excretion was not due to specific renal damage, nor was it caused by increased ingestion of the vitamin. It was claimed that excretion only took place in hypovitaminosis C, and that it could be checked by giving ascorbic acid. Lindqvist [1937] found that in pneumonia the excretion of vitamin A varied from 228 to 3060 I.U. daily before crisis, falling to zero after crisis. Urinary excretion was associated with a lowered vitamin A content of the blood, which returned to normal after crisis. Gaehtgens [1937] reported the presence of vitamin A in urine from 8 out of 31 cases of normal pregnancy. After large doses of vitamin A had been given it was found in the urine in 19 cases.

In chronic nephritis Hedberg & Lindqvist [1938] observed constant or irregular excretion of vitamin A in 23 out of 25 cases. In contrast with the finding in pneumonia the vitamin A content of the blood was often high. In livers taken at autopsy the reserves were frequently low, but bore no relation to

[1] A preliminary communication on this subject has been given to the Biochemical Society [Lawrie *et al.* 1938].

[2] Rockefeller Research Fellow.

the levels found in the blood. Large doses of vitamin A, ascorbic acid or pyramidone did not effect urinary excretion. Of 26 patients examined by Grant [1938], 15 showed spontaneous excretion of vitamin A. These included patients suffering from carcinoma of the gall bladder, cirrhosis of the liver, icterus, hemeralopia, chronic nephritis, nephrosis, diabetes and pneumonia. The mean vitamin A content of the blood in this group was much lower than in the remaining group of 11 cases which did not excrete vitamin A, although the same diseases were often found in both groups. The low values for the excreting cases could not, however, be ascribed directly to loss in the urine, since some cases which were not excreting vitamin A showed equally low levels in the blood. The cholesterol content of the blood was independent of either the level of vitamin A in the blood or of the presence or absence of urinary excretion of vitamin A.

The finding of Hedberg & Lindqvist [1938] that reduction of vitamin A in the blood does not necessarily occur in cases of renal disease is supported by Catel [1937; 1938] who found that the blood vitamin A remained normal in children with nephritis accompanied by urinary excretion of the vitamin. This worker made the surprising claim, which has been confirmed in the present work, that vitamin A is present in the urine of the normal dog. Vitamin A was not detected in the urine of rabbits. Chevallier & Manuel [1938] have claimed that vitamin A may be present in the urine of guinea-pigs which have been given large doses of the vitamin.

The urinary excretion of vitamin A in skin diseases has been investigated by Marchionini [1938]. Out of 75 cases of non-tuberculous skin disease vitamin A was excreted spontaneously in the urine in 7 cases, and in 15 cases after massive doses of vitamin A. In 27 cases of tuberculous skin diseases, mostly lupus, spontaneous excretion was found in 1 case, excretion after dosing in 7 cases. In 14 cases of syphilis, with and without skin lesions, vitamin A was excreted spontaneously in 2 cases and after dosing in 9 cases. In subjects excreting vitamin A the blood vitamin A was sometimes low, but not always. No evidence of kidney damage was obtained, but in many cases the galactose test revealed reduced liver efficiency. The excretion of vitamin A appeared to be secondary to this injury. Erythema induced by ultra-violet irradiation caused reduction in the carotene and vitamin A contents of the blood, but did not result in the urinary excretion of vitamin A.

Recently, evidence to connect urinary excretion of vitamin A with injury or blockage of the reticulo-endothelial system has been advanced by Thiele and his colleagues. Thiele & Seedorf [1939] reported that vitamin A is occasionally excreted by patients undergoing treatment for gonorrhoea and tertiary syphilis. The excretion did not appear to depend on injury to the liver or kidneys, but was frequent in subjects treated by bismuth or by infection with malaria. Similar findings were recorded in a later paper by Thiele & Nemitz [1939]. The efficiency of the reticulo-endothelial system in subjects showing urinary excretion of vitamin A was tested by Thiele & Klodwig [1939]. When Congo red was injected into the blood stream its rate of disappearance was usually abnormally slow, indicating impairment of the reticulo-endothelial cells.

Experimental

Part I. *The examination of human and animal urines for vitamin A*

Method. 100 ml. of urine were shaken with successive additions of 5 ml. of saturated aqueous KOH, 10 ml. of ethanol and 100 ml. of ether. With urines containing much vitamin A smaller volumes of urine and reagents were some-

times taken. The ether layer was washed with water, evaporated and dissolved in 1 ml. of $CHCl_3$. Vitamin A was estimated by the $SbCl_3$ method. To convert blue units into International Units a factor of 0·6 has been used [Moore, 1937].

Normal human subjects. Urine was collected from 6 subjects, of ages 7 months to 41 years. In no instance could even a trace of vitamin A be detected. The vitamin was not excreted by some of the same individuals when suffering from severe colds. One subject took halibut-liver oil to the extent of 30,000 I.U. of vitamin A daily for 2 months without causing urinary excretion. Another ingested 200 g. of raw ox liver, again with negative results.

Pathological human subjects. 83 urines from various medical cases were examined. The results are shown in Table 1. Vitamin A was excreted most frequently and in highest concentration in pneumonia. Next in order of frequency

Table 1. *The urinary excretion of vitamin A in human diseases*

The numbers after each disease which are not included in brackets give the vitamin A content per 100 ml. of urine for individual cases. The numbers in brackets give the corresponding daily excretions of vitamin A.

Frequent excretion of vitamin A

Respiratory diseases: Pneumonia 0, 0, 6 (126), 12 (37), 19, 60, 68, 105, 160 (3200), 250, 340 (1500), 340, 400, 400, 450, 480. Empyema 0, 3, 68. Pulmonary tuberculosis 0, 018 (pyonephrosis + calculus). Asthma 0, 15. *Kidney diseases*: Chronic nephritis 0, 0, 0, 0, 12 (96), 14, 18, 26, 37, 52. Sub-acute glomerular nephritis 34 (187). Acute nephritis 0, 0. Prostate uraemia 41. Nephrosis 68. Nephrosis with osteomyelitis 8. Albuminuria 0.

Infrequent or absent excretion of vitamin A

Rheumatic diseases: Rheumatic fever 0, 0, 9. Acute rheumatism 0. Muscular rheumatism 0. Rheumatoid arthritis 0, 0. Fibrositis 0. *Heart diseases*: Cardiac failure 0, 0. Aortic regurgitation 0, 0, 0. Hypertension 0. Coronary thrombosis 0. *Diabetes*: 0, 0, 0, 4, 5. *Cancer*: Obstructive jaundice due to carcinoma at head of pancreas 0. Carcinoma of colon 0. *Cerebral tumour*: 0, 0, 0, 0. *Liver diseases*: Cirrhosis of liver 0. Sub-acute atrophy 0. *Thyroid diseases*: Rheumatic carditis and hyperthyroidism 0. Thyrotoxicosis 0. *Miscellaneous*: Plummer-Vinsen syndrome 0. Post-encephalitic Parkinsonism 0. Disseminated sclerosis 0. Splenic anaemia 0. Haemophilia 0. Pernicious anaemia 3. Haematemesis 0, 0. Abdominal tumour of unknown origin 0. Duodenal ulcer 0. Epilepsy 0. Abortus fever 0. Osteoarthritis 0. Dermatosis 0. Pyrexia of unknown origin 0.

of excretion and concentration was chronic nephritis. We have not noticed more frequent or pronounced excretion in any one type of pneumonia or chronic nephritis than in others. Excretion was fairly common in other respiratory and kidney diseases. In our remaining 44 cases of general diseases we found only three instances of the excretion of small amounts of vitamin A. No significance need be attached to our failure to confirm the positive results obtained by previous workers for cancer and skin diseases in view of the small number of cases which we have examined. In pneumonia we found, in agreement with other workers, that the excretion of vitamin A ceased abruptly when the crisis had been passed. In chronic nephritis the excretion was usually continuous and permanent.

Pregnancy in women. Samples of urine from 32 cases of pregnancy were obtained through the kindness of Drs H. R. Youngman and W. B. Hedgcock. The duration of pregnancy had varied from 16 to 38 weeks. Of 30 subjects who received no special vitamin A therapy, 24 were described as normal. In only one instance was a trace of vitamin A detected in the urine. No vitamin A was detected in urine from two cases of urinary infection. Vitamin A was present in

the urine in one out of four cases of albuminuria, to the extent of 12 I.U./100 ml. Urine from a normal subject who had received 12,000 I.U. daily of a vitamin A concentrate for a month, and from a subject, recently recovered from urinary infection, who had received halibut-liver oil to the extent of 70,000 I.U. daily for 1 week, gave negative results.

These results suggest that the urinary excretion of vitamin A in pregnancy, at least in this country, is less prevalent than the data of Gaehtgens [1937] would indicate for Germany.

The excretion of vitamin A by animals

Dog. Catel [1937; 1938] has reported that vitamin A is present in the urine of the normal dog. We have confirmed this, using urine from a healthy male fox terrier. Many specimens were examined and they were always positive. The concentration of vitamin A varied between 90 and 450 I.U./100 ml. of urine, determined colorimetrically, the latter value being equal to the highest concentrations we have found in urine from patients with pneumonia. The diet of the dog normally included household scraps, biscuits and occasional liver. When the animal was deprived of liver and greens for 10 days and then restricted for 3 days to a diet low in vitamin A (biscuits only) there was no diminution in the excretion of vitamin A. The restoration of a mixed dietary for the next 3 days, in conjunction with the administration of vitamin A (120,000 I.U. daily as halibut-liver oil) was associated with a marked decrease in the concentration of vitamin A in the urine. While we are inclined to attribute this anomalous observation to mere coincidence, it seems clear that vitamin A as ingested cannot pass freely into the urine, and that the amount of vitamin A excreted is determined by factors other than the supply immediately available from the diet.

Rabbits. Catel [1937; 1938] could not detect vitamin A in the urine of rabbits. We have confirmed this finding for normal adult rabbits, and have found only small traces of the vitamin in the urine after infection with sputum from a human patient with pneumonia.

Our first rabbit was given a subcutaneous injection of a suspension of sputum in the groin. The concentration of vitamin A in the urine during the next 2 days was only *ca.* 10 I.U./100 ml. The animal then died, the autopsy revealing red and grey hepatization of the lungs and acute congestion of the spleen, liver and kidneys. A second rabbit was similarly treated and died after 3 days. Bacterial examination showed the presence of pneumococci in the blood and lungs. Only a trace of vitamin A (2 I.U./100 ml.) was present in the urine. In order to rule out the possibility that the failure of the first two rabbits to excrete large amounts of vitamin A had been due to the inadequacy of the reserve in their liver a third rabbit was given a diet rich in green vegetables, with 5 drops of cod-liver oil per week for nearly 2 months. During the last 5 days before the injection of sputum a total of 25,000 I.U. of vitamin A was given as halibut-liver oil. No vitamin A could be detected in the urine either before or after the injection. At autopsy the liver was found to contain a total of 17,000 I.U. of vitamin A, equivalent to 210 I.U./g.

In view of the report of Thiele & Seedorf [1939] that treatment with bismuth may cause the excretion of vitamin A, rabbit no. 3, before receiving the injection of pneumococci, was given a single injection of 25 mg. of metallic bismuth as a watery suspension (Bismoid, Eli Lilly and Co.). No excretion of vitamin A could be detected during the next 48 hr., although the presence of bismuth in the urine could readily be demonstrated by chemical tests.

Rats. Vitamin A was not detected in the urine of either normal or diseased rats. The injection of pneumonia sputum into rats neither affected the health of the animals nor caused excretion of vitamin A. An attempt to infect the animals with pus taken from the kidney of another rat was also unsuccessful. Repeated bismuth injections over a period of 8 days did not cause excretion of

the vitamin. No vitamin A was found in urine collected from rats having severe degeneration of the epithelium of the convoluted tubules of the kidneys caused by prolonged deficiency of vitamin E [Martin & Moore, 1939], nor in the urine of a rat in the final stages of a fatal respiratory disease of spontaneous origin.

Biological and spectroscopic confirmation of the presence of vitamin A in urine. Previous workers have relied on the $SbCl_3$ test for the demonstration of vitamin A in urine extracts. Boller *et al.* [1937] reported that the absorption band in this reaction is at about 620 mμ, the position characteristic of the vitamin. We have confirmed this observation. It seemed desirable nevertheless to rule out the possibility that the $SbCl_3$ reaction might be simulated by some unidentified excretory product. An extract of urine from a patient with empyema, who excreted about 70 I.U. of vitamin A/100 ml. according to the $SbCl_3$ test, was therefore examined in the ultra-violet spectrophotometer. A shallow band at 328 mμ was observed. Biological tests were made with extracts of urines from a patient with pneumonia and from the healthy dog, which contained 180 and 150 I.U. of vitamin A/100 ml. respectively. In each instance increases in weight were observed when the extracts were dosed to rats at levels equivalent to about 8 I.U. per day.

Part II. *The state of combination of vitamin A in urine*

Normal human urine is almost free from lipoids [Neuberg, 1911]. In disease fatty material may appear in the urine in one or other of the following forms: (1) fat globules or tallow-like particles floating on the surface, (2) a milky emulsion (chyluria), (3) fatty concretions and needle-shaped crystals, (4) constituents of cells rich in fat, (5) a lecithin-globulin complex giving rise to fine turbidity. Most of the urines containing vitamin A which we have examined do not fall into any of the above groups. Some were as clear as normal urine. Others were turbid, but on filtration many gave filtrates still containing vitamin A, yet as clear as freshly voided normal urine. Since vitamin A is strictly fat-soluble the question arises as to what the form of combination or dispersion in urine may be. The first possibility to be examined was that vitamin A, although insoluble in water, might be soluble in normal or pathological urine.

The insolubility of vitamin A in normal urine. 2 ml. of halibut-liver oil were added to 100 ml. of normal urine. The mixture was shaken vigorously for $\frac{1}{2}$ hr. and then filtered through four wet Whatman no. 44 filter papers in succession. No vitamin A could be detected in the filtrate. Similarly, vitamin A extracted from pneumonia urines could not be dissolved in normal urines. For example, 40 ml. of a pneumonia urine showing a strong $SbCl_3$ reaction was extracted with ether and alcohol and the residue, after evaporation, was shaken with 50 ml. of normal urine and filtered through a wet filter paper. No vitamin A could be detected in the filtrate.

The solubility of vitamin A in some pathological urines. Pathological urines were next tested to determine whether they would dissolve vitamin A. In general it was found that urines containing vitamin A were capable of taking up more vitamin when shaken with halibut-liver oil (see Table 2).

Attempts were next made to find how vitamin A is held in solution in urine. Most urines containing vitamin A also contained heat-coagulable protein. No quantitative relation between the amounts of protein and vitamin A excreted could, however, be traced. In one case of nephrosis the daily outputs of globulin, albumin and vitamin A were measured for a month, but no correlation was apparent.

Table 2

Subject	Description	Vitamin A in urine I.U./100 ml. Before shaking with H.-L. oil	After shaking with H.-L. oil
Human	Normal	0	0
,,	Pyrexia (unknown origin)	0	18
,,	Pneumonia	0	85
,,	Pneumonia early	60 (clear)	190 (turbid)
,,	Pneumonia	370 (clear)	510 (clear)
,,	Proteinuria	68	1650 (turbid)
Dog	Normal	60	225

Note. A solution of 0·5% bile salts, when treated with halibut-liver oil in the same way as these urines, took up 425 I.U. of vitamin A/100 ml.

Precipitation of vitamin A with heat-coagulable protein. When the protein in such a urine was coagulated by heating, all the vitamin A was found in the coagulum. This may be merely an adsorption effect, since when concentrates of the vitamin in the water-soluble state (see below) are heated much of the vitamin escapes precipitation. This suggests that removal of the vitamin by heat coagulation of the protein is only complete when conditions for adsorption are favourable, i.e. much protein and relatively little vitamin A in the solution.

Urines of subjects with pneumonia show the highest vitamin A contents and yet often contain little heat-coagulable protein. In one instance 30 I.U. of vitamin A per 100 ml. were present in such a urine which contained no heat-coagulable protein but gave reactions for proteose and peptone. We have never obtained a preparation containing vitamin A in watery solution which did not give the protein colour reactions.

The concentration from urine of vitamin A in the water-soluble state

The following experiments were performed with a view to devising a method for the concentration of the vitamin.

Precipitation by basic lead acetate. 45 ml. of a urine giving a strongly positive test for vitamin A were treated with 5 ml. of a saturated solution of basic lead acetate and filtered. The filtrate gave a negative test for vitamin A and the residue a strongly positive test. The vitamin A could not be recovered in watery solutions, however, by gassing with H_2S.

Precipitation with acetone. Addition of acetone to urines containing vitamin A yielded precipitates containing no vitamin A. This procedure therefore gave promise of effecting a partial separation of the water-soluble vitamin A complex. Much destruction, however, appeared to occur and this procedure was abandoned.

Adsorption on kaolin. The process of adsorption on kaolin at an acid *p*H and elution at an alkaline *p*H has been much used in the purification of enzymes, and its applicability to our problem was tested on the assumption that the vitamin A might be combined as a lipoprotein. A urine containing 19 I.U. of vitamin A/100 ml. was first used. 500 ml. were shaken up with a little kaolin and the *p*H adjusted to 4·0. The kaolin was filtered off and re-suspended in water, the *p*H adjusted to 9·0 and the suspension filtered after standing for 2 hr. 71% of the vitamin A was found to have been adsorbed and subsequently eluted and 16% not to have been adsorbed.

This experiment was then repeated with a urine containing 375 I.U./100 ml. Only 19% of the vitamin A was found in the eluate. In a similar experiment using a urine containing 375 I.U./100 ml., 16% was found in the eluate after a first adsorption and 23% of the remainder after a second treatment.

These experiments indicate that while adsorption by this method and subsequent elution is efficient with low concentrations of vitamin A, it is not serviceable for the comparatively high concentrations found in pneumonia.

Adsorption on charcoal. Merck's blood charcoal was used. With urine containing 450 I.U. of vitamin A/100 ml. 33% of the vitamin disappeared after shaking with the charcoal, and of this almost none could be eluted. Thus this procedure is not likely to be of use except for the decolorization of strong preparations obtained by other means.

Concentration in vacuo. Little loss of vitamin A occurred during this procedure, which was often used in making concentrates.

Dialysis. The vitamin A was retained on dialysis through parchment paper against water. Parchment paper sacs permitted rapid removal of salts, the dialysis being first carried out against running tap water and finally against distilled water.

Procedure for concentration of vitamin A in the water-soluble state

Our most effective procedure was as follows. The urine was first fully saturated with ammonium sulphate. The precipitate formed was filtered off on a Büchner funnel, the paper carefully removed and the funnel washed out. The paper was then replaced, and about 100 ml. of distilled water were drawn through the paper. This water was poured repeatedly through the paper till no more of the brown precipitate was removed; the process was repeated with 2–3 changes of water. The extracts were then combined and concentrated *in vacuo* to a volume of 20–50 ml. This concentrate was dialysed in a parchment sac, first against running tap water and later against distilled water, until it no longer gave a precipitate with $BaCl_2$ and HCl.

Concentrates prepared in this manner were brown and usually contained a little heat-coagulable protein which could be removed by boiling with a drop or two of 5% acetic acid and filtering. The precipitate of heat-coagulable protein contained some vitamin A. The filtrate had the following properties. (1) Vitamin A, a strongly positive $SbCl_3$ test. (2) Mercuric nitrate test (Millon's reaction for tyrosine): strongly positive. (3) Aldehyde reaction of tryptophan (glyoxylic test): probably positive, but the colour was obscured by the brown colour of the preparation. (4) Arginine reaction (Sakaguchi, with α-naphthol): moderately strongly positive. (5) Biuret reaction: positive, violet. (6) Molisch reaction (for carbohydrate): negative.

From the above findings it appears that vitamin A, as it occurs pathologically in urine, is associated with a non-heat-coagulable protein fraction.

The solution of vitamin A by protein derivatives. In view of the association of vitamin A in the urine with this nitrogenous fraction, it was of interest to find to what extent vitamin A from halibut-liver oil could be obtained in watery solution by means of protein derivatives. A 5% solution of 'Difco' proteose-peptone, salt and halibut-liver oil were ground together in a mortar and filtered twice through wet filter papers. Although only poor emulsification was obtained by this means, the filtrate showed a positive $SbCl_3$ test of low intensity. These experiments were not carried out quantitatively, but since the halibut-liver oil used contained about 60,000 I.U. of vitamin A/1 ml., only a very small fraction of the vitamin A present in the oil was extracted into the watery phase. A concentration similar to that occurring in the urine in many cases of chronic nephritis was, however, readily obtained.

Concentration of vitamin A per unit of urinary lipoid. With all urines containing vitamin A only small amounts of fatty material could be obtained by the

usual procedure of adding KOH and alcohol and extracting with ether, a treatment which would not be expected to cause saponification of triglycerides. When only small quantities were extracted the amounts of fat obtained were too small to be weighed without elaborate precautions to ensure drying to constant weight. Large amounts of urine were, however, collected from a few subjects for the purpose of vitamin D estimations (Table 4). Sufficient lipoid to permit accurate weighing was obtained in one instance: the vitamin A concentration was about 23 I.U./mg., which is about 10 times greater than that found in cod-liver oil and would be a reasonable value for human liver fat. The urine examined showed only moderately heavy excretion of the vitamin, and much more concentrated extracts, probably up to 200 I.U./mg., have been obtained in other instances.

Part III. *The vitamin A content of human kidneys*

Experiments with animals have indicated that the kidneys are one of the most important sites of distribution of the relatively small amounts of vitamin A that are not absorbed by the liver. Evidence of the concentration of vitamin A in the human kidney may therefore help in our understanding of the excretion of the vitamin in the urine in disease. The data on specimens obtained at autopsy, given in Table 3, were obtained during an investigation of the vitamin A

Table 3. *The vitamin A content of the kidney at autopsy*

Individual values, in I.U./g., for the vitamin A contents of the liver and kidney were determined at autopsy on subjects dying from accidental death and various groups of diseases.

Accidental death														
Liver	15	120	150	150	150	150	180	180	300	300	300	375	450	750	750
Kidney	0·6	1·2	0	0·6	1·2	1·2	1·2	1·2	1·2	2·4	6	0·3	2·4	3·6	4·8
Diseases of alimentary tract														
Liver	6	60	90	90										
Kidney	0	0	0	2·4										
Organic heart disease														
Liver	22	37	45	45	60	60	60	60	60	90	90	90	120	120	
Kidney	0	0	0	0	0	0	0·6	0·6	1·2	0	0	1·2	0	1·2	
Respiratory diseases														
Liver	6	12	15	30	37	337								
Kidney	0	0	0	0	0	2·6								
Nephritis														
Liver	0	1·5	3	3	9	9	9	15	45	60	75	135	375	
Kidney	0	0	0	0	0	0	0	0	0	0·3	0	0·6	6	
Renal infections														
Liver	15	15	30	37	45	135	135	375						
Kidney	0·6	0·6	0	0	0	0·6	3·6	0·3						

reserves of the liver [Moore, 1937]. It will be seen that in accidental death 14 out of 15 specimens gave positive results in the $SbCl_3$ test under the particular conditions of concentration adopted. In disease the ranges of the vitamin A reserves of the liver were lower, and the proportion of kidney specimens giving positive $SbCl_3$ tests was much smaller. Thus in respiratory diseases negative results were obtained for 5 out of 6 specimens, in nephritis for 10 out of 13 specimens.

Part IV. *The excretion of vitamin D in urine*

In view of the frequent association of vitamin D with vitamin A in liver oils experiments were undertaken to find out whether this vitamin can be excreted in urine. Large volumes of urine (in one instance 44 l.) were collected from one normal and three pathological subjects, some of whom were given large doses of calciferol. To extract the fraction which would be expected to include vitamin D, if present, the following procedure was used. The urines were either concentrated by evaporation, or the protein was separated by precipitation. The products so obtained were digested with alkali, alcohol was added and the lipoid fractions were extracted with ether. After evaporation the extracts were diluted with arachis oil and dosed to rats which had been kept on Steenbock's diet No. 2965 (yellow maize 76%, NaCl 1%, $CaCO_3$ 3%, wheat gluten 20%) until severe rickets had developed.

The unit taken in adjusting the level of dosing was 1 day's excretion by the subject, which was divided into even doses spread over the curative period. In one case the dose corresponded to 6 days' excretion by the subject. The duration of the experiment with the dog, which received 20,000 I.U. of calciferol per day was, however, sufficient to permit dosing at the level of only about half the daily output. The degrees of curing were assessed by X-ray examination, a solution of calciferol being used as a standard. Results are given in Table 4.

Table 4. *The excretion of vitamin D in urine*

Subject	Disease	Approx. daily excretion of vitamin A I.U.	Daily dosage of calciferol I.U.	Total daily excretion of lipoids mg.	No. of days urinary output from which test dose was extracted	Daily excretion of vitamin D I.U.
Human	None	—	9,000	8	1	0
,,	Chronic nephritis	350	0	3	1	0
,,	Osteomyelitis with nephrosis	800	9,000	35	1	20
,,	Nephrosis	30	0	10	6	1
Dog	None	400	20,000	0	½	0

No vitamin D could be detected in urine from the normal human subject or from the dog, although large doses of calciferol had been given. Negative results were also obtained in the patient with chronic nephritis who received no doses. 20 I.U. of vitamin D were excreted daily by the patient with osteomyelitis who had been dosed with calciferol, and a trace by the patient with nephrosis, who was not dosed.

Discussion

Selectivity of the excretion of vitamin A. Vitamin A is associated in urine with only minute amounts of lipoids and its excretion appears to be highly selective. If we take round figures of 100 I.U./100 ml. for vitamin A, and 600 mg./100 ml. for the lipoid content of human plasma, then the concentration of vitamin A related to lipoid will be about 0·17 I.U./mg. The exact value will vary considerably in individual cases, both in health and disease, but will remain of the same order of magnitude. In pathological urine the concentration per unit of lipoid may be much higher. In the present work an extract containing 23 I.U./mg. of lipoid was obtained, and it seems probable that much higher concentrations are frequent. The concentration per unit of lipoid in the urine is therefore at least 100 times greater than in blood. The selective nature of the excretion of vitamin A

is further emphasized by the virtual absence of vitamin D from the urines of the human subject and the dog given calciferol.

The conditions determining urinary excretion of vitamin A. A full explanation of the excretion of vitamin A in urine is made difficult by the diversity of the conditions under which it occurs. Thus in the dog heavy excretion is found in normal health. In the human subject vitamin A is absent from the urine in health, but present in some pathological conditions. In the rat we have failed to detect excretion of the vitamin even in disease. No single theory seems capable of explaining all the facts. Even the attractive suggestion that excretion of vitamin A results from injury to the reticulo-endothelial system breaks down in the case of the dog. It is difficult moreover, to understand why, if this system as a whole is involved in the storage of vitamin A, the spleen and bone marrow should not contain large amounts.

Two generalizations, however, seem possible. (1) Urine which contains vitamin A differs from normal urine in its ability to take up more vitamin A, from halibut-liver oil, in a form sufficiently dispersed to pass through damp filter paper. This property appears to be associated with the presence of protein in the urine. It may be noted that Schneider & Weigand [1937, 1, 2] and Hedberg & Lindqvist [1938] have remarked on the tendency of urines containing vitamin A to form emulsions with ether. (2) The two most prominent diseases in which vitamin A is excreted in the urine, chronic nephritis and pneumonia, are among those in which low ranges of reserves of vitamin A have been found in the liver at autopsy [Moore, 1937].

These generalizations clearly do not necessarily hold good in an opposite direction. Urines containing much protein, as in acute nephritis, are often devoid of vitamin A, although they possess the property of taking up the vitamin when shaken with halibut-liver oil. The reserves of vitamin A in the liver at autopsy are sometimes low in diseases in which vitamin A is seldom present in the urine.

Abnormal solubility relationships. The cause of the urinary excretion of vitamin A by pathological human subjects can be pictured most readily as arising from alterations in the relative powers of the liver, blood, kidneys and urine to dissolve or absorb the vitamin. In the normal human subject, as in other vertebrates, vitamin A is preferentially absorbed and retained by the liver to a remarkable degree. The distribution of vitamin A between this organ and the blood stream must be considered as an equilibrium, since in the human subject the vitamin A content of the liver never attains more than some 10% of the concentration which may be assumed, from experiments with many different animals, to represent saturation. In the dog this equilibrium is changed in the direction of increased solution of vitamin A in the blood by the ingestion of alcohol [Clausen *et al.* 1940], but otherwise we know little of the factors controlling the distribution in health.

The relative distributions of the vitamin in the human being in health, pneumonia and chronic nephritis, as far as they can be assessed from data obtained for the various organs and fluids on different groups of subjects, are summarized in Table 5. In both pneumonia and chronic nephritis the amount of vitamin A held by the liver is reduced. In pneumonia the absorptive powers of the blood and kidneys are also reduced and a high concentration of vitamin A passes into the urine, together with abnormal metabolites capable of holding it in solution. In chronic nephritis, the liver reserve is usually more seriously reduced than in pneumonia. The level of vitamin A is often raised in the blood, but lowered in the kidney. The concentration of vitamin A in the urine is usually lower than in pneumonia. These differences may possibly be explained by the

Table 5

	Vitamin A (I.U./100 g.)			
	Liver	Blood serum	Kidneys	Urine
Normal	22,000	70	190	0
Pneumonia	6,300	27	40	190
Chronic nephritis	2,500	120	50	16

Liver values are medians found by Moore [1937]. 'Normals' refer to cases of accidental death. Blood values are means from data of Lindqvist [1938]. Kidney values from means of data given in Table 3. Urine values from means of data given in Table 1.

inefficiency of the kidneys in chronic nephritis, which might lead to the accumulation in the blood of substances which increase the solution of vitamin A.

The occasional occurrence of proteinuria without excretion of vitamin A indicates that all urines capable of dissolving vitamin A do not necessarily contain it. It seems probable that in the human subject at least, functional abnormality of the liver must be involved before excretion of vitamin A takes place.

Quantitative aspects. In pneumonia the amount of vitamin A lost in the urine may exceed the reputed daily requirement of 3000 I.U. Since at a given time the kidneys of a typical healthy adult must contain about 500 I.U. of vitamin A and the blood 2000 I.U. it is clear that excretion cannot be long maintained from these sources without their replenishment from elsewhere. Surprisingly large amounts of vitamin A may be obtained in an invalid diet composed largely of milk, and further amounts might be mobilized during the use of depot fats. In view of the fall of vitamin A in the liver, however, it seems probable that the vitamin A excreted in the urine is derived from this organ. It is important, however, to realize that the daily excretion in pneumonia, say 3000 I.U., is small compared with the liver reserve of 330,000 I.U. typical of the healthy adult. The reduction of the reserve in pneumonia to a median value of 94,000 I.U. cannot all be ascribed to urinary excretion, and much vitamin is presumably lost without leaving any trace of its fate.

In chronic nephritis the daily excretion is usually lower. As in pneumonia, therefore, the reduction of the median value for the liver reserve to 38,000 I.U. cannot be accounted for quantitatively by the urinary excretion.

Summary

1. Vitamin A could not be detected by means of the $SbCl_3$ test in samples of normal human urine, even from subjects who had received large doses of vitamin A. The reported frequent occurrence of vitamin A in human urine during pregnancy was not confirmed. Carotene was never found in significant amount in urine of any description.

2. The frequent excretion of vitamin A in urine in some human diseases, particularly pneumonia and chronic nephritis, was confirmed. In pneumonia the daily excretion sometimes exceeded 3000 I.U., the reputed daily requirement for this vitamin. The rate of excretion in chronic nephritis was generally less.

3. The presence of large amounts of vitamin A in the urine of the healthy dog was confirmed. Vitamin A was not found in the urine of normal or diseased rats. Not more than traces were detected in the urine of a cat or of normal or diseased rabbits. The attempted blockage of the reticulo-endothelial system by injections of bismuth in the rat or rabbits did not cause excretion of the vitamin.

4. The positive results by the $SbCl_3$ methods were confirmed in one urine by the presence of the absorption band at 328 mμ characteristic of vitamin A, and in two others by biological tests.

5. Some human urines containing vitamin A were clear, others were turbid, but some of these could be filtered to complete clarity without the removal of vitamin A. Protein, not necessarily heat-coagulable, was always present. Urines originally containing vitamin A took up still more vitamin A when shaken with halibut-liver oil. Normal human urine did not do this, but those which contained protein without vitamin A did.

6. Traces of vitamin A were found regularly in kidneys taken at autopsy in cases of accidental death. In disease the amounts present were generally reduced. Under the conditions of the test, negative results were obtained in 5 out of 6 cases of respiratory disease, and in 10 out of 13 cases of nephritis.

7. Vitamin D was absent from, or present only in traces in, the urine of human subjects or the dog, even when large doses of calciferol had been ingested. When 9000 I.U. of calciferol were given daily to a diseased subject with spontaneous excretion of vitamin A, only 20 I.U. daily of vitamin D were excreted in the urine.

8. The excretion of vitamin A in urine is highly selective, the concentration of vitamin per unit of lipoid being much greater in urine than in blood. It may be significant that chronic nephritis and pneumonia, the two diseases in which urinary excretion of vitamin A is most frequent, are among those in which very low ranges of reserves have been found in the liver at autopsy. The amount of vitamin A lost in the urine cannot, however, account fully for the diminution of the liver reserves in these diseases.

Our thanks are due to Dr L. J. Harris for his valuable criticism, to Dr B. McArdle for collaboration in the clinical parts of this work, and to the Staff of Addenbrooke's Hospital for permission to investigate the cases under their care.

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92. THE ABSORPTION AND EXCRETION OF 'MINOR' ELEMENTS BY MAN

1. SILVER, GOLD, LITHIUM, BORON AND VANADIUM

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(*Received 23 June 1941*)

Our knowledge of the biological principles underlying the excretion of minerals, whether physiological, pharmacological or pathological, is surprisingly meagre. Even the route by which many of them leave the human body is not yet known, much less the rate at which they do so. Within recent years iron has been shown to be excreted much less readily than was previously supposed [McCance & Widdowson, 1938], and not by the large intestine [Nicolayson, 1935; Welch *et al.* 1936], but there are many problems underlying the excretion of calcium and other elements in the same periodic group [McCance & Widdowson, 1939, 1, 2]. The introduction of radioactive elements into the physiologist's armamentarium has stimulated interest in this subject and provided some data of great value. The use of these 'marked' elements, however, is still in its infancy, and has by no means displaced older methods. For some purposes it never can. Accordingly, as opportunities presented themselves of studying the excretion of various metals, it was natural to do so, and the analytical technique, together with some of the results, form the substance of this paper.

Subjects and methods

The subjects of these experiments have been patients, and normal persons. The former were used for the studies of Ag and Au. The latter had submitted themselves to prolonged balance experiments in order to solve other problems in mineral metabolism, and it was while they were thus engaged that their excretion of V, Li and B was investigated. The technique of these balance experiments has been so fully described by McCance & Widdowson [1941] that nothing further need be said.

The minerals were introduced into the body in various ways which will be described in the appropriate sections.

The analyses were done spectrochemically. A Hilger medium quartz spectrograph (E. 3) was used with Ilford Auto-Filter (ortho) and Rapid Process Panchromatic plates. The electrodes, the purest obtainable, were supplied by Messrs Adam Hilger Ltd., with an analyst's report of their impurities. The light source was an oxy-coal gas flame, a low voltage D.C. arc or a high voltage A.C. spark. Ag was estimated by a modification of the Ramage flame method, full details of which have been published [Kent, 1940]. The filter paper standards contained 100, 10, 5, 2, 1, 0·5 and 0·1 μg. of Ag. The other elements were determined by comparing their lines with those of an internal reference element. Mg or Fe was generally selected for this purpose, and if so its concentration in the analytical material was estimated chemically. Bi and Mo were used as the reference elements for Sn (see Table 1). They were added in equal quantities both to the

'unknown' and to the 'standard' solutions, neither of which contained any until this was done.

For the spark, a pair of graphite electrodes (6·5 mm. diameter) was used (Hilger lab. no. 12313), the upper of which was bored longitudinally with a 1 mm. diameter hole. One end of this electrode was pointed, while the other end was fitted with a rubber teat, by means of which the liquid for analysis was drawn up into the electrode. The lower electrode had a flat end, and the spark gap was about 2 mm. wide. The electrodes were connected through a condenser (0·005 μ farad) to the secondary circuit of a transformer (generating 15,000 V.), the primary circuit of which was connected through a resistance to the A.C. mains. The spark was about 25 cm. from the slit of the spectrograph, and the exposure was usually 20 sec. For the arc a pair of pure copper electrodes was used, of which the upper (5 mm. diameter, Hilger lab. no. 11184) was pointed, the lower (7 mm. diameter, Hilger lab. no. 10300) flattened. A small quantity of residue from the evaporated solutions (*vide infra*) was placed on the lower electrode. The arc was struck by bringing the electrodes together, and then separating them until the current was approximately 1·8 A. This current was maintained for 60 sec. The arc was connected in series with the D.C. mains and with a resistance which cut down the potential to about 60 V.

The intensity of the lines in the spectra was measured by a microphotometer which was constructed in the Department of Physical Chemistry, Cambridge, where all the spectrographic work was carried out. A framework held the plate vertically, and permitted a coarse up-and-down motion and a fine sideways motion. Light from a 12 V., 60 W., lamp was focussed on the plate by means of two lenses, and an image of the line was focussed by a third lens on the slit of a photoelectric cell. The latter was connected to a non-recording mirror galvanometer. The most sensitive line of the element to be estimated was usually chosen for microphotometric measurements. The choice of internal reference element was governed by the distribution of lines in the spectrum as it was essential to have the line of the reference element fairly close to the line of the element to be estimated. In Table 1 are listed the elements estimated and the method of excitation, the internal reference element and the pairs of comparison lines used for photometric determinations.

Table 1. *Summary of quantitative spectrographic methods*

			Comparison pairs of lines	
Element estimated	Process	Internal standard	Element estimated	Standard
Lithium	Arc	Magnesium	Li 3232	Mg 2803
Boron	Spark	Iron	B 2496·7	Fe 2493
Vanadium	Spark	Magnesium	V 3102	Mg 3097
		Iron	V 3102	Fe 2783
Silver	Ramage flame	—	Ag 3280	—
			Ag 3383	—
Gold	Arc	Magnesium	Au 2676	Mg 2795
Manganese*	Spark	Iron	Mn 2606	Fe 2607
Nickel*	Spark	Iron	Ni 2394	Fe 2388
		,,	Ni 2394	Fe 2384
Cobalt*	Spark	Iron	Co 2378	Fe 2379
		,,	Co 2388·9	Fe 2388·6
Tin*	Arc	Bismuth	Sn 3034	Bi 2997
		Molybdenum	Sn 3034	Mo 2911

* The absorption and excretion of these elements will be discussed in a subsequent paper.

When the density of a line was being measured with the microphotometer, the plate was moved very slowly with the fine screw so that the line passed completely across the slit of the photoelectric cell. The spot light from the galvanometer was watched and the maximum deflexion noted. A reading was also recorded for the lighter ground adjacent to each line measured. The intensity of the line of the internal reference element was measured before passing on to another spectrogram on the same plate.

Food, urine and faeces were prepared for chemical analysis as described by McCance & Widdowson [1941], and were supplied in duplicate for spectrochemical analysis in the form of a dilute HCl extract of the ash. The ash extracts of the foods and urines were prepared for analysis by the spark method by making the concentration of the Fe in them approximately equal to that in the ash extracts of the faeces. For this purpose 0·8 ml. of an Fe solution, containing 0·5 mg. of Fe/ml., was added to 9·2 ml. of the ash extracts of the food, and 1 ml. of the same Fe solution to 9 ml. of the ash extracts of the urines. Unless this had been done, erroneously high results were obtained for Mn both in the foods and urines, and as a precaution, therefore, the Fe concentration was similarly adjusted when other elements were being estimated. In the arc method the Fe concentration was adjusted as outlined above, the Bi and Mo were added if Sn was being determined, and the solutions were evaporated to dryness. Two exposures were made of each ash extract or of the dry residue prepared from it. Hence, quadruplicate spectrograms were taken of the food, urine and faeces in each experimental period.

For each element to be estimated a series of standard solutions was prepared containing varying quantities of that element, but the same quantities of the internal reference element. The concentrations of the reference elements in these serial standards were 0·05 mg. of Bi and of Mo/ml., 0·4 mg. of Mg/ml., and 0·05 mg. of Fe/ml. To each was also added the same quantity of a 'background' solution containing Na, K and Ca, the purpose of which was to give these solutions a composition similar to that of the unknowns. From the microphotometric readings of these standard solutions a series of ratios, Intensity of the element to be estimated/Intensity of reference element, was obtained. When the logarithm of these ratios was plotted on squared paper against the logarithm of the percentage of the element to be estimated, straight lines or smooth curves were obtained. From these 'working curves' the log of the percentage of the unknown in any ash extract could be obtained by working out the log of the intensity ratio from the microphotometric results, and reading off the answer from the graph.

Results

Silver

The study was made on a woman who was suffering from generalized argyria caused by washing out her nose for many years with an organic silver preparation. She was nearly, but not quite as pigmented as the case shown in Harker & Hunter's [1935] coloured plate. Her Ag metabolism was followed for three analytical periods, each of 7 days in length. The food, urine and faeces were prepared for analysis as described by McCance & Widdowson [1941]. The results of the balances are given in Table 2. It will be noted that the patient was excreting very little of the silver with which she appeared to be so saturated, and this result is in keeping with the clinical observations, all of which go to show that the pigmentation, once acquired, does not fade rapidly, if at all. The small negative balances which are shown may not even be physiological in the true

Table 2. *The intake by mouth and the excretion of silver by a patient with argyria*

Week	Food mg./week	Urine mg./week	Faeces mg./week	Total excretion mg./week	Balance mg./week
1	0·05	0	1·3	1·3	−1·25
2	0	0	1·5	1·5	−1·5
3	0·7	0	2·3	2·3	−1·6

sense of the term, for the mucous membranes of the patient's mouth and nose were deeply pigmented, and the proctoscope revealed similar, although less deep, staining in the visible parts of the rectum and lower bowel. The whole alimentary canal, therefore, may have been lined by cells containing Ag. Desquamation of some of these cells may have been the cause of the negative balances.

Gold

Gold is given therapeutically by injection, and attempts have been made from time to time to discover the organs in which it is deposited [Gerlach, 1935; Koppenhöfer, 1936; Leulier & Béruard, 1938; Leulier & Payre-Ficot, 1934, 2; Lumière & Julliard, 1930], and the route by which it is excreted [Arloing *et al.* 1932; Leulier *et al.* 1937; Leulier & Béruard, 1938; Leulier & Payre-Ficot, 1934, 1]. Most of this work appears to have been done in France and Germany. Both men and laboratory animals have been investigated. All species do not necessarily react in quite the same way [Leulier & Payre-Ficot, 1934, 2], but it has been established that the drug may cause nephritis, that it is excreted very slowly, and that the more soluble compounds, such as Solganol B and Myochrysine, are excreted predominantly by the kidney, whereas the more insoluble, such as Lipaurol and Myoral, in so far as they are excreted at all, are passed out mainly by the gut [Leulier & Payre-Ficot, 1934, 1].

The present work was carried out on a woman who was being treated for rheumatism with injections of Solganol B oleosum, an oily suspension of aurothioglucose which is said to be water-soluble and to contain 50% of Au [Leulier & Payre-Ficot, 1934, 1; Gerlach, 1935]. There were two analytical periods, each of 7 days in length, and in each the patient received 50 mg. of gold by intramuscular injection. 500 mg. of Au had already been given before the study began. The results, which are shown in Table 3, corroborate those of the French authors.

Table 3. *Excretion of gold after intramuscular injection*

Week	Food mg./week	Urine mg./week	Faeces mg./week	Total excretion mg./week	% of the total excreted in the urine
1	0	5·2	1·5	6·7	78
2	0	7·1	2·0	9·1	78

Some [Arloing *et al.* 1932; Leulier & Payre-Ficot, 1934, 1, 2; Leulier *et al.* 1937] have found a rather higher proportion of these soluble salts excreted in the urine, but the present partition is of very much the usual order. It is evident, however, that some Au is genuinely excreted by the gut. How it reaches the intestine is unknown, and seems to be a matter for investigation.

Lithium

Practically no papers on the absorption and excretion of lithium salts have been found since Good's [1903] article on the pharmacology of this metal. Good studied the oral and subcutaneous administration of toxic doses of LiCl to cats

and dogs, and found that, however given, they produced vomiting, enteritis and death. In spite of these signs of gastro-intestinal excretion, and the finding of lithium in the saliva and gastric juices, the metal was eliminated mainly, although incompletely, by the kidney. This process did not cause nephritis. Table 4 shows the lithium balances of two men and one woman before, during

Table 4. *Lithium balances before, during and after ingesting soluble lithium salts*

Subject	Week	Intake by mouth mg./week	Lithium in urine mg./week	'Total' lithium excreted (urine + faeces) mg./week	Lithium balance mg./week	Lithium recovered mg.
N. K.	1	11·1	4·7	9·7	+ 1·4	
(Male)	2	6·3 + 250	218	229	+27	236
	3	7·9	15	21	−13	
E. W.	1	17	4·3	17	± 0	
(Female)	2	17 + 250	218	233	+34	258
	3	16	46	58	−42	
R. M.	1	18	4·5	19	− 1	
(Male)	2	16 + 250	195	208	+58	247
	3	17	60	72	−55	

and after a time in which 250 mg. of LiCl were taken by mouth. The salt was given after meals, over a period of 3 days, during the second week of a 3-week metabolism experiment. N. K. took the Li in the early part of the week, the others in the last 3 days, and this explains why E. W. and R. M. excreted more of the administered Li in the third week. It is evident that soluble Li salts added to a diet are readily absorbed and quantitatively excreted in the urine. In N. K.'s second week there were 5 mg. more lithium in the faeces than there were in the solid food. This must have come from the medicinal 250 mg., but apart from this trifling exception, none of the administered Li seemed to reach the faeces.

The present results, therefore, differ somewhat from those of Good, but the experimental conditions were totally different. In the light of modern knowledge it is easy to explain the excretion by the gut of small amounts of any metal which can replace Na in the extracellular fluids, and of quite large amounts if the metal in its passage to the lumen of the gut produces a gastroenteritis and diarrhoea.

If now the first week of each person's experiment is inspected, it will be seen that the urine contained only 25–50% of the total amount excreted. This suggests a very poor absorption of Li from natural diets. Table 5 shows the metabolic data in more detail, and also gives three other 'balances'. The brown

Table 5. *The intake and excretion of lithium when 40–50% of the total calories in the diets were supplied by white bread, or by brown*

Subject	Diet	Week	Food mg./week	Urine mg./week	Faeces mg./week	Li absorbed (food − faeces) mg./week
N. K.	White bread	1	11·1	4·7	5·0	6·1
R. M.	,,	1	12·5	4·6	6·6	5·9
	,,	2	11·4	4·2	6·8	4·6
	,,	3	12·4	6·5	7·3	5·1
E. W.	Brown bread	1	16·9	4·3	12·4	4·5
R. M.	,,	1	17·7	4·5	14·0	3·7

bread diets contained more Li than the white because the 92% flour had much more in it than the 69%. The figures obtained by analysis were 0·42 and 0·17 mg./100 g. respectively. It is, however, strange that the Li was absorbed so badly from both diets, and particularly so from the brown bread. Potassium was absorbed much more freely [McCance & Widdowson, 1941]. Admittedly, calcium and magnesium were not well absorbed from the 92% flour, but this was found to be due to their precipitation as phytates. The poor absorption of Li cannot be explained in this way, for lithium phytate is soluble. There was, moreover, very little phytic acid in the white flour diets, the lithium in which was also relatively poorly absorbed. It is possible that the Li in the branny parts of wheat is in very close association with the cellulose particles and never really goes into solution in the intestine. Li has physiological properties in plants which suggest that this may be so, for it has been found that Li does not return from the senescent wheat leaf as potassium does, but remains with the calcium *in situ* [Mason & Maskell, 1931; Kent, 1941]. Its manner of fixation in these leaves is not yet known. It is evident, however, that for some reason the Li in natural foods may be very much less freely absorbed than soluble Li salts, even if the latter have been taken with food.

Boron

Plants were first recognized to contain B in 1857 [Brenchley, 1927] and this element is now accepted as a constituent of all vegetable tissues. Consequently, it is an inevitable component of our daily diet, and, apart from this physiological aspect of the matter, boric acid has had such a checkered career as a food preservative that some experiments have been carried out to determine the fate of the B which is naturally present in foods, and of boric acid when taken by mouth. Something is known of this latter. Röst [1905] and Wiley [1907] both studied the excretion of boric acid after doses of 3 g. and upwards had been given, and concluded that 82–100% was quickly excreted in the urine. The faeces were never an important avenue of excretion, and although small amounts were found in the sweat, this also was quantitatively unimportant. Oddly enough, neither author considered the B naturally present in food, but this was probably due to their technical limitations. The upper half of Table 6 shows the results which were obtained when two normal women each took (in all) 352 mg. of B as boric acid during the 3rd, 4th and 5th days of a 28-day metabolism experiment. 40% of the calories of their diets were derived from white flour. It will be seen that 93–94% of the B was recovered during the first week, which demonstrates its rapid absorption and metabolism, and confirms the results of Röst [1905], Wiley [1907] and Presnell & Brill [1937]. It will also be noted from the last 2 weeks of the metabolism experiment that the B in natural foods was metabolized in very much the same way. The lower half of Table 6 shows the B balances for the same two women over a period of 21 days when 40% of their calories were derived from flour of 92% extraction. 100 g. of the white flour contained 0·045 mg. of B. 100 g. of the 92% flour contained 0·16 mg., a figure which agrees quite well with those of Bertrand & De Wall [1936] for whole wheat and cereals generally. It may seem strange, therefore, that the intakes were not higher during the brown bread than they were during the white bread experiments. The explanation is that even the 92% flour accounted for only 4·0 mg., or about 5% of the B in the week's food. The greater part of the B in these diets seems to have been introduced by the large amounts of fruit, particularly plums and greengages, which were being consumed [Allen & Tankard, 1904]. It is not thought that any of the foods had had any boric acid added to

Table 6. *Boron balances on white bread diets after ingesting boric acid*

Subject	Week	Intake by mouth mg./week	Boron in urine mg./week	Total boron excreted (urine + faeces) mg./week	Boron balance mg./week	Boron recovered
		Boron balances on white bread diets.				
E. W.	1	89 + 352	408	417	+ 24	351
	2	81	100	104	− 23	
	3	64	53	57	+ 7	
	4	88	86	92	− 4	
A. M.	1	113 + 352	435	445	+ 20	332
	2	142	123	129	+ 13	
	3	111	100	104	+ 7	
	4	138	124	135	+ 3	
		Boron balances on brown bread diets.				
E. W.		75	62	68	+ 7	
A. M.		96	100	109	− 13	

them. These results on the whole are in keeping with those of Löhnis [1936] and of Bertrand & De Wall [1936], who stated that cereals contained very much less B than fruits and vegetables.

Vanadium

Vanadium has been employed in the therapy of syphilis. Proescher *et al.* [1917] investigated some of its actions and reported that the hexa- were less toxic than the tetra-vanadates, but that there was not a large margin between the therapeutic and the toxic doses. They also reported that the drug was not 'cumulative'. When 12·5 mg. of V_2O_5 were taken by mouth daily for 12 days, 87·6% of it was recovered in the faeces, and 12·4% in the urine. Thus all the administered V was recovered, but no attempt was made to find out how much of the 87% represented unabsorbed V, and how much re-excreted.

In the present experiments two normal men, whose metabolism was being studied for three successive weeks, were given six intravenous injections of sodium tetravanadate dissolved in normal saline during the second week; 3–5 min. were taken over each injection. The results are shown in Table 7. It

Table 7. *The excretion of vanadium after intravenous injections of Na tetravanadate*

		Intake		Excretion			
Subject	Week	Food mg./week	Injection mg./week	Urine mg./week	Faeces mg./week	Total mg./week	Vanadium recovered
N. K.	1	0	18	10·4	1·4	11·8	16·8 mg. ≡ 93% of the dose
	2	0	0	5·0	0·0	5·0	
C. B.	1	0	24	13·2	1·3	14·5	21·2 mg. ≡ 89% of the dose
	2	0	0	6·8	0·0	6·8	

will be seen that most of the V was excreted during the week in which it was given, and that very little remained in the body by the end of the subsequent week. During the week of injections some 10% of the excreted V was found in the faeces, but it is clear that V which has once been absorbed is mainly eliminated by the kidney. The greater part of the oral dose found by Proescher *et al.* [1917] in the faeces should therefore be regarded as unabsorbed V and this element can be said to be one of those which is not readily absorbed when taken by mouth as the vanadate but, once absorbed, is excreted almost entirely by the kidney.

SUMMARY

Balance experiments have shown that:—

1. A patient with severe generalized argyria excreted less than 2 mg. of silver per week, all of it in the faeces.

2. A patient who had received 550 mg. of gold intramuscularly, excreted 6·7 and 9·1 mg. in two successive weeks, 78% of it in the urine.

3. Soluble lithium salts taken orally by normal persons were rapidly and completely excreted in the urine, but the Li in natural foods was poorly absorbed. Only 25% of the Li in a brown bread diet was excreted in the urine.

4. Boron taken by mouth as boric acid was rapidly excreted in the urine. B in foods was much more readily absorbed than Li, 92% of it appearing in the urine.

5. 81% of the vanadium, which had been administered intravenously as sodium tetravanadate to normal persons in six daily doses, was excreted in the urine by the 7th day after the last injection. 9% was excreted in the faeces in the same time.

The authors are grateful to Prof. Norrish for placing a spectrograph at their disposal and to Dr W. C. Price for some very valuable technical advice. They have also to thank Drs J. H. Sheldon and F. B. Parsons for allowing their patients to be investigated. The work could never have been undertaken, without the help and co-operation of the subjects, and also of Miss B. Alington and Miss E. M. Widdowson. The greater part of the expenses were covered by a grant made by the Medical Research Council.

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93. MECHANISM OF BIOLOGICAL NITROGEN FIXATION

7. MOLECULAR H_2 AND THE pN_2 FUNCTION OF AZOTOBACTER[1]

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(*Received 3 July 1941*)

A PREVIOUS report [Wyss & Wilson, 1941] demonstrated that H_2 inhibits fixation of N_2 by *Azotobacter*. Because of the apparent uniqueness of this type of inhibition, such a demonstration furnishes strong evidence that in the symbiotic and free-living N_2 fixation processes the mechanism of fixation is very similar if not identical. Further studies described in this paper provide additional support for this important conclusion.

Methods

Details of the methods used were described by Wyss & Wilson [1941]. Briefly, 25 ml. cultures of *Azotobacter* were grown under different atmospheres in litre bottles provided with a device which maintained the pO_2 constant (0·2 atm.) during the experiment. Under these conditions fixation remains logarithmic for 24–48 hr.; hence the specific rate constant of fixation, $k = d \ln (a+y)/dt$, can be readily estimated from the slope of the line obtained by plotting the log of the total N content against time. In this equation a is the initial N content of the cells and y is the increase after t hr. With cultures of *Azotobacter vinelandii*, k consistently reaches the surprisingly high values of 0·080–0·125 corresponding to a total fixation of 20–30 mg./100 ml. in 24 hr. Somewhat lower values were noted with *Az. chroococcum*. Harvests were made every 10–12 hr. often in duplicate so that each k value is based on 4–6 separate determinations. Respiration measurements were made in the Warburg apparatus by the usual technique, and micro-N determinations by the method of Johnson [1941].

The pN_2 function

A fundamental property of any enzyme system is the dependence of the rate of reaction on the concentration of substrate. Burk [1930] found that the rate of N_2 fixation by *Azotobacter* was a function of the pN_2 between 0·05 and 5 to 10 atm., being directly proportional to the pN_2 between 0·05 and 0·5 atm. Apparently these conclusions were based almost entirely on microrespiration experiments in which H_2 was used as the diluent gas. It follows then that the pN_2 function discussed by Burk [1934] is not the true one but is a complex of the effects of both N_2 and H_2. Failure to recognize the inhibition by H_2 probably explains two characteristics of the pN_2 function which Burk thought surprising, viz. that fixation hardly occurred until a pN_2 of 0·05 atm. was present, and that

[1] This research was aided by a grant from the Rockefeller Foundation. Technical assistance was furnished by the personnel of the Works Progress Administration: Official Project No. OP 65-1-53-2349.

in comparison with other enzyme-catalysed gas reactions, the Michaelis constant, K_{N_2}, was extremely high (0·21 atm.).

The pN_2 function of *Azotobacter* was determined in the presence of different 'inert' gases—argon, helium, and hydrogen. Results from the macro total N experiments are summarized in Table 1. In each experiment a 'control' was

Table 1. *Nitrogen fixation by* Azotobacter *as a function of the* pN_2 *in presence of He, A and* H_2 (macro-N experiments)

	Relative values of k under different pN_2 in atm.								
$k_{air}=100$	0·012	0·025	0·05	0·1	0·15	0·20	0·25	0·30	0·40
Helium									
0·095	—	—	—	93	—	96	—	103	—
0·095	—	—	—	90	—	100	—	97	—
0·064	—	70	79	93	—	96	—	—	—
0·083	70	80	82	91	—	98	—	—	—
0·071	40	—	—	76	—	—	—	—	—
0·090	52	56	—	—	—	—	95	—	—
0·125	—	—	—	85	93	—	95	—	—
0·060*	—	—	76	88	98	—	—	—	—
Argon									
0·037*	—	—	—	88	—	100	104	102†	—
0·043*	—	—	—	—	—	99	—	99	102
0·110	—	—	—	—	—	—	95	96†	97
0·040*	—	—	—	—	91	—	97	—	—
Hydrogen									
0·070	—	—	—	19	—	74	—	—	93
0·073	—	—	—	32	—	74	—	80	90
0·071	—	—	19	39	62	—	86	94†	—
0·052	—	—	—	—	—	—	66	67†	89‡
0·108	—	—	—	—	—	—	82	92†	96‡
0·043*	—	—	—	—	—	68	—	81	95
0·110	—	—	—	—	—	73	—	86	91
0·125	—	—	—	38	64	—	83	—	—
0·040*	—	—	—	—	46	62	74	—	—
0·060*	—	—	21	43	57	—	—	—	—

* *Az. chroococcum*; all other *Az. vinelandii*. † 0·35 atm. ‡ 0·45 atm.

In Tables 1, 2 and 3 the figures under k_{air} are the actual specific rate constants; those opposite a given k_{air} are the relative values for the indicated atmospheres.

Table 2. *Nitrogen fixation by* Azotobacter *as a function of the* pN_2 *in absence of an inert gas* (macro-N vacuum experiments)

	Relative k values	
$k_{air}=100$	0·2	0·15
0·064	108	—
0·070	101	—
0·073	101	—
0·070	—	95
0·037*	101	—
0·081	—	92
0·052	—	92
0·083	93	—
0·108	—	95
0·110	91	—
0·040*	101	—
0·125	94	—
0·060*	—	90†
0·072	—	94
0·101	—	93

* *Az. chroococcum*; all others *Az. vinelandii*. † 0·1 atm.

included at some value of the pN_2, usually, 0·2 atm., in which no inert gas was added but instead the cultures were grown under a partial vacuum; the pO_2 for such cultures was kept constant at 0·2 atm. by supplying pure O_2 through a column of mercury. These values are given in Table 2.

The averages of the data in these two tables, together with values from a few miscellaneous experiments, are shown in Fig. 1, which emphasizes the striking

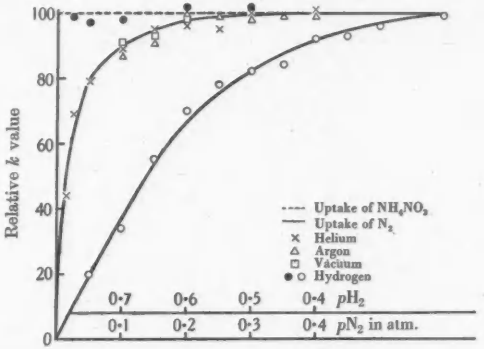

Fig. 1. The pN_2 function of *Azotobacter* based on macro total N experiments. This figure is compiled from over 100 separate estimations of the rate of fixation in different gas mixtures. The individual points shown are the means of 3–7 estimations; each of these is based on analyses of 4–6 cultures.

difference in the effect of the pN_2 in the presence and absence of H_2. If He, A, or a vacuum replaces the abstracted N_2, no consistent change in the rate of fixation is observed until the pN_2 is lowered to approximately 0·15 atm.; the rate then decreases slowly with the pN_2 so that at 0·01 atm. the value of k still is about 50% of the maximum. In the presence of H_2, however, a significant reduction in the rate of fixation is noted at a pN_2 of 0·45 atm. (pH_2=0·35 atm.). As more H_2 is added, the rate drops so rapidly that at a pN_2 of 0·05 atm. (pH_2=0·75 atm.) fixation is barely detectable in 48 hr. That this effect of H_2 is specifically associated with the N_2 fixation reaction is indicated by the data in Table 3 which show that the uptake of NH_4NO_3-N is independent of H_2 in the atmosphere.

Table 3. *Assimilation of NH_4NO_3 by* Azotobacter *as a function of the* pN_2 *in the presence of* H_2 (macro-N experiments)

	Relative values of k for different pN_2 in atm.				
k_{air}=100	0·025	0·05	0·10	0·20	0·30
0·115	—	103	101	99	102
0·056	—	102	104	111	97
0·109	99	100	97	98	—
0·093	—	99	94	98	—
0·031*	—	81	93	97	—

* *Az. chroococcum*; all others *Az. vinelandii*.

The values obtained with H_2 in these macro-N experiments which lasted 24–48 hr. are in reasonable agreement with those reported by Burk who estimated uptake of free or combined N by microrespiration measurements over

periods of 4–8 hr. It was believed desirable, however, to check at least the critical values indicated by the curves in Fig. 1 by the microrespiration technique. Results from typical experiments are summarized in Table 4. In these experiments duplicate or triplicate flasks were taken of each treatment, and the best

Table 4. *Nitrogen fixation by* Azotobacter *as a function of the* pN_2 *in the presence of A, He, and* H_2 (microrespiration experiments)

Diluent gas	Treatment: k values for different pN_2				Air control
	0·05 atm.	0·10 atm.		0·15 atm.	
H_2		0·133, 0·132 } 0·133			
He	0·219, 0·219 } 0·219	0·229, 0·252 } 0·240		0·263, 0·248, 0·232 } 0·248	0·306
	0·3 atm.	0·4 atm.	0·5 atm.	0·6 atm.	
H_2	0·208, 0·211 } 0·210	0·245	0·260, 0·243 } 0·252	0·261, 0·265 } 0·263	
He	0·278, 0·303 } 0·290				0·277, 0·287 } 0·282
	0·4 atm.	0·5 atm.		0·6 atm.	
H_2*	0·359, 0·364, 0·353 } 0·359	0·350, 0·367, 0·368 } 0·361		0·399, 0·403, 0·389 } 0·397	0·440, 0·442, 0·422 } 0·431
	0·01 atm.	0·025 atm.		0·05 atm.	
He	0·104, 0·103, 0·105 } 0·104	0·143, 0·144 } 0·144		0·163, 0·163 } 0·163	0·290, 0·293, 0·277 } 0·287
	0·01 atm.	0·025 atm.		0·05 atm.	
He	0·136, 0·137, 0·138 } 0·137	0·155, 0·161, 0·155 } 0·157		0·193, 0·185 } 0·189	0·275, 0·286, 0·270 } 0·277
		0·05 atm.		0·10 atm.	
H_2 (NH_4NO_3)		0·278, 0·254 } 0·266		0·260, 0·263, 0·264 } 0·262	0·290, 0·262, 0·265 } 0·272

* *Az. chroococcum*; all others *Az. vinelandii*.

straight line for each flask was determined by the usual statistical procedure. In a given experiment the variances, s^2, for the slopes of the different lines were shown to be homogeneous; therefore these could be pooled and a common variance for the experiment calculated. When this was done, it was readily demonstrable that growing cultures in an atmosphere with a pH_2 of 0·2 atm. or more decreased significantly the mean value of k in comparison with the air control. It appears then that when the $pN_2 : pH_2$ ratio is high, the microrespiration technique is more sensitive for detection of inhibition than is the macro-N method. The final experiment cited in Table 4 demonstrates that the effect of H_2 is specifically concerned with the fixation system since the values of k for uptake of NH_4NO_3-N (estimated by the increase in rate of respiration of *Azotobacter* with time) are independent of the pH_2. As was observed in the macro-N experiments whenever helium was used as the diluent, the rate of fixation was significantly lowered only after the pN_2 was reduced to about 0·15 atm. Even at a pN_2 of approximately 0·01 atm. the rate of fixation reached

40–50 % of the value in air. As is evident in Fig. 2, the pN_2 curves based on the microrespiration data are very similar in the presence and absence of H_2 to the corresponding functions derived from the macro-N experiments.

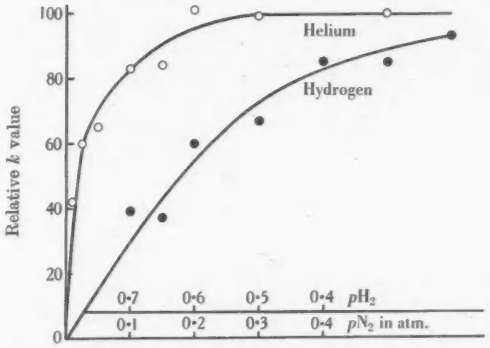

Fig. 2. The pN_2 function of *Azotobacter* based on microrespiration experiments. Each point represents the mean of 2–6 determinations in separate experiments; in each experiment duplicate or triplicate flasks were taken.

In some of the respiration experiments several flasks of each treatment were taken, and periodically one was removed for micro-N determinations. The values of k together with the necessary statistical constants for estimation of the significance of observed differences are given in Table 5. The micro-N determinations are less accurate than the respiration data, but the two sets of results

Table 5. *Nitrogen fixation by* Azotobacter *as a function of the* pN_2 (micro-N experiments)

Composition of gas mixture*			Respiration			Micro-N		
pN_2 atm.	pHe atm.	pH_2 atm.	k value	N	S_k†	k value	N	S_k†
0·8	—	—	0·273	18	0·0138	0·148	4	0·0131
0·15	0·65	—	0·233	18	0·0115	0·149	4	0·0051
0·15	—	0·65	0·139	18	0·0095	0·080	4	0·0150
0·8	—	—	0·226	18	0·0104	0·188	6	0·0099
0·3	0·5	—	0·210	27	0·0087	0·193	8	0·0163
0·3	—	0·5	0·171	19	0·0085	0·117	8	0·0112
0·8	—	—	0·232	18	0·0087	0·187	5	0·0104
0·2	0·6	—	—	—	—	0·206	5	0·0267
0·1	0·7	—	0·205	18	0·092	0·168	4	0·0147

* $pO_2 = 0{\cdot}2$ atm. in all mixtures.

† ~~$S_k = S_y \sqrt{\Sigma (x - \bar{x})^2}$~~. To test difference between two values of k, calculate $S' = \sqrt{(S_{k_1}{}^2 + S_{k_2}{}^2)}$. Then $t = \dfrac{k_1 - k_2}{S'}$ with $N_1 + N_2 - 4$ degrees of freedom.

$S_k = 2{\cdot}303 \; S_b = 2{\cdot}303 \sqrt{\dfrac{s^2}{\Sigma (x - \bar{x})^2}}$

parallel one another sufficiently to conclude that increase in O_2 uptake probably serves as a reliable index for the estimation of N_2 fixation. In general the k values based on respiration are consistently higher than those based on total N, but the relative values do not differ materially so that the conclusions drawn from either type of data would be the same. Specifically, they show that reduction

of the pN_2 to 0·1–0·2 atm. causes only a small decrease in the rate of N_2 fixation by *Azotobacter* in the presence of helium but a marked decrease in the presence of H_2.

Nature of the inhibition

The type of inhibition by H_2—whether competitive or non-competitive—was determined by comparing the rates of N_2 fixation at different values of the pN_2 and pH_2. As discussed in detail by Wilson *et al.* [1941], if the concentration of substrate (pN_2) is kept constant the relative rate of fixation in the presence of inhibitor (k/k_i) is a linear function of the concentration of inhibitor (pH_2) with an intercept of unity. If the inhibition is competitive, the slopes of the lines obtained when k/k_i is plotted against the pH_2 for different values of the pN_2 are significantly different. The method is illustrated in Fig. 3 in which the data from Exps. II and III of Table 6 are used.

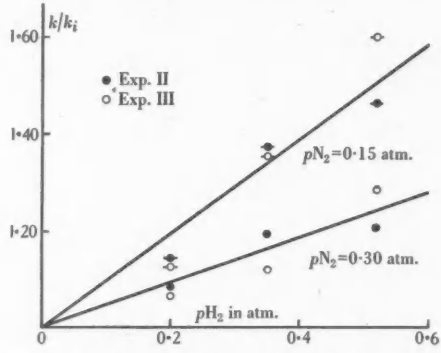

Fig. 3. Competition of H_2 with N_2 in *Azotobacter* N fixation experiments. The significant difference in the slopes of the lines obtained when k/k_1 is plotted against the pH_2 for different values of the pN_2 indicates that the inhibition is competitive.

Table 6. *Nature of inhibition by H_2* (macro-N experiments)

Exp.	pN_2 atm.	Slope of line	t value for difference in slopes
I	0·2	0·4170	2·97
	0·4	0·2235	
II	0·15	0·9118	5·60
	0·30	0·4383	
III	0·15	1·0709	5·27
	0·30	0·4959	

Since each line contributes 2 degrees of freedom, t must be at least 2·78 for a significant difference (19 : 1 odds). As this value was exceeded in all the experiments, it is concluded that the slopes of the lines differed significantly; therefore the inhibition is competitive.

Reversibility of inhibition

The reversibility of the inhibition by H_2 as well as its complete removal by combined N was demonstrated by a macro-N experiment with *Az. vinelandii* in which the conditions under which assimilation of N_2 occurred were altered

during the experiment. The treatments and results are shown in Fig. 4. The values of k were: air throughout, 0·080 (line 1); H_2 throughout, 0·037 (line 6). When cultures originally under air were shifted at the end of 32 hr. to the atmosphere containing H_2 (line 3), the rate of fixation decreased to that of H_2 throughout. Likewise, when after 32 hr. cultures in the H_2 atmosphere were transferred to air (line 5), the rate increased to that of cultures kept in air

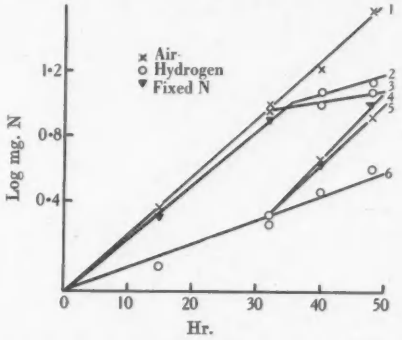

Fig. 4. Reversibility of H_2 inhibition of N fixation by *Azotobacter*. (H_2 indicates: pH_2, 0·65 atm.; pO_2, 0·20 atm.; pN_2, 0·15 atm.)

1. Air throughout.
2. 10 mg. fixed N under H_2.
3. Air to 32 hr., change to H_2.
4. H_2 to 32 hr., 20 mg. N added, continued under H_2.
5. H_2 to 32 hr., change to air.
6. H_2 throughout.

throughout the experiment. Moreover, if excess NH_4NO_3-N was added to cultures kept under the H_2 atmosphere, the inhibition by H_2 was removed even though incubation continued under the same atmosphere (line 4). Finally, if a small quantity of combined nitrogen was added initially to the cultures, H_2 did not inhibit until all fixed N was utilized (line 2).

Comparison of effects of H_2 on fixation by red clover and Azotobacter

Additional evidence that H_2 affects the symbiotic and asymbiotic N fixation systems in a similar manner was obtained by an experiment in which red clover plants and *Azotobacter* cultures were kept in the same environment. The plants were grown in 9 l. pyrex bottles under the gas mixture: pO_2, 0·2 atm.; pN_2, 0·3 atm. In one series (*Vacuum*) no other gas was supplied, in a second (*Hydrogen*) H_2 was added to 1 atm. The substrate was a N-poor sand plus N-free Crone's solution. In each series one-half of the plant cultures were inoculated with *Rhizobium trifolii* 209; the remainder were supplied with NH_4NO_3. After 10 weeks 200 ml. tubes containing 50 ml. of Burk's medium inoculated with *Azotobacter vinelandii* were placed in representative bottles of each series. NH_4NO_3 was added to the medium whenever the tube was placed in a red clover culture furnished with this source of N. The *Azotobacter* tubes were wrapped in light-tight paper so that the bottles could be kept in a greenhouse. The bottles were then stoppered, and the experimental atmospheres were replaced. The *Azotobacter* cultures metabolized the sucrose in the medium to produce CO_2 for the plants; the plants assimilated this with production of O_2 thus keeping the pO_2 reasonably constant. A type of 'symbiosis' resulted which maintained the N-fixing systems of the *Azotobacter* and red clover plants in the same gaseous environment.

After one week the bottles containing both red clover and *Azotobacter* cultures were harvested, and the experiment was repeated the following week with the remaining bottles. During this second week, the temperature of the greenhouse was raised from 24° to 28°, and helium was added to 1 atm. in the bottles of the *Vacuum* series. The following data summarize the results; the values given (mg. total N per culture) are the means of triplicate samples:

Treatment	*Azotobacter*		Red clover	
	N_2	NH_4NO_3	N_2	NH_4NO_3
I. First week:				
Vacuum	6·88	5·10	54·7	70·0
Hydrogen	3·70	5·60	34·5	62·8
II. Second week:				
Helium	13·10	9·42	68·9	102·2
Hydrogen	9·90	9·35	44·1	103·1

Although the conditions were not ideal for fixation of N_2 by *Azotobacter* (low temperature, small surface exposed), the type of result was identical with that observed in the experiments made under more optimum conditions. The data of this combination trial confirm the conclusion based on the extensive observations made with the separate N-fixing systems: fixation of N_2 by both inoculated red clover plants and *Azotobacter* is inhibited by H_2 but assimilation of NH_4NO_3-N is not affected.

Discussion

The results presented in this paper together with those previously reported provide strong support for the view that the enzyme systems responsible for the fixation reaction in inoculated red clover plants and in the free-living *Azotobacter* are identical. Before discussing the significance of this conclusion, we shall briefly analyse the major evidence in its favour.

The pN_2 function. The manner in which the rate of reaction changes with concentration of substrate is very similar for both systems. If the curves in Figs. 1 and 2 are compared with that for the symbiotic system [Wilson, 1940], a close resemblance is noted. In both cases the rate of reaction is independent (95–100% of maximum) of the pN_2 until the latter is lowered to about 0·15 atm., and the apparent Michaelis constants (K_{N_2}) are of the same order of magnitude. Estimation of the K_{N_2} for *Azotobacter* from the results of the macro total N experiments by the method of Lineweaver & Burk [1934] gave a value of 0·012 atm.; the less extensive microrespirometer data indicated a K_{N_2} of 0·015 atm. Great precision, however, is not claimed for these values since the number of experiments on which the estimates are based is not large, but they are sufficiently accurate for our purposes. In our previous studies with red clover a K_{N_2} of approximately 0·05 atm. was obtained for the symbiotic system. This figure is very likely a maximum value since the technical conditions which must be used in the plant studies may cause the rate of diffusion of the gases into the nodule to affect the determination of the K_{N_2}. Diffusion may likewise influence the estimates of the K_{N_2} for the *Azotobacter* system, but in view of the close agreement between the values obtained with quiescent macrocultures and the vigorously shaken microcultures, this appears less likely. It seems probable that the values of the K_{N_2} for both systems are fairly low (*ca.* 0·01–0·02 atm.), are of the same order of magnitude and do not differ greatly from those of other enzyme-catalysed gas reactions.

Inhibition by H_2. Not only does H_2 inhibit both systems, which in itself is rather unusual, but the quantitative aspects of the inhibition are similar. If

the rate of N_2 fixation by red clover plants is plotted against the pH_2 in the atmosphere, a straight line results [Wilson *et al.* 1938], whereas the corresponding curve for the *Azotobacter* system appears to be hyperbolic (Figs. 1 and 2). This difference in the type of curve is reflected in the K_{H_2} values of the two systems. On the assumption that the K_{N_2} for *Azotobacter* is 0·01 atm., the data from the three experiments of Table 6 give a mean value of 0·08 atm. for the K_{H_2}. The K_{H_2} for the symbiotic system in red clover plants is 0·14 atm. on the basis of a K_{N_2} of 0·05 atm. [Wilson, 1940]. That is, the ratio of the affinity of the N_2-fixing system for N_2 to that for H_2 is 8 in *Azotobacter* and 3 in red clover. In the symbiotic system, however, the values of the Michaelis constants may be affected to some extent by the relative rates of diffusion of the gases into the nodules so that this comparatively small (2 to 3-fold) difference may not be significant. In addition, the fact that the inhibition is competitive and is readily reversible in both instances affords strong support for the view that the two systems respond to H_2 in an identical manner.

The identification of the symbiotic and asymbiotic N_2-fixing enzyme systems through comparison of their physical-chemical characteristics has important implications for the general field of biological mechanisms as well as for the specific problem of the mechanism of biological N fixation. This application of the physical-chemical approach to a problem of biological mechanism demonstrates its usefulness for investigation of enzyme systems which are not readily separated from the living cell. It should be noted that, *a priori*, one could not conclude that the responsible enzyme systems in these two biological agents are similar since obvious differences exist in the symbiotic and asymbiotic methods for N_2 fixation—differences which may or may not reflect fundamental dissimilarities in the mechanism of the reaction. The question naturally arises: do other biological agents which fix N_2—*Clostridia*, for example—possess a mechanism similar to that of *Azotobacter* and inoculated red clover plants? Investigations similar to those described in this and previous reports should provide the answer. Since the *Clostridia* liberate H_2 during breakdown of carbohydrates, it will be of interest to learn whether this cellular reaction affects the concurrent one of N_2 fixation.

Summary

Based on macro total nitrogen rate experiments, the pN_2 function of *Azotobacter* is characterized by the complete independence of the rate of fixation of the pN_2 when the latter exceeds 0·2 atm. Below this value the rate of fixation decreases slowly; a K_{N_2} of approximately 0·01 atm. is indicated. In the presence of H_2, however, a decrease in the rate of fixation is observed when the pN_2 is reduced to 0·45–0·5 atm. The decrease in the rate with pN_2 (increasing pH_2) proceeds very rapidly so that at a pN_2 of 0·05 atm. fixation is barely detectable. This effect of H_2 is not observed with cultures supplied with NH_4NO_3; hence the effect is specific for the nitrogen fixation process.

The pN_2 functions of *Azotobacter* in the presence of different diluent gases determined by microrespiration experiments are practically identical with those determined by the macro total nitrogen experiments.

The inhibitory action of H_2 is reversible and competitive. The K_{H_2} value is approximately 0·08 atm., i.e. the affinity of the N_2-fixing system for N_2 is 8 times that for H_2.

These physical-chemical properties of the N_2-fixing system in *Azotobacter* are very similar to those we have previously established for red clover plants

inoculated with the specific root nodule bacteria. It is therefore concluded that the responsible enzyme systems in these diverse biological agents—free-living *Azotobacter* and symbiotic plant plus bacteria—are identical.

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